Monograph on **Benzolic Acid & Sodium Benzoate**-Volume 2 8/31/72

MONOGRAPH
ON
BENZOIC ACID
AND
SODIUM BENZOATE
VOL. II
DOCUMENTS

TR-72-1552-07

Submitted Under:
Contract No. FDA 72-104

August 31, 1972

INFORMATICS INC.
6000 Executive Boulevard
Rockville, Maryland 20852

Drug Biotransformation Interactions in Man II: A Pharmacokinetic Study of the Simultaneous Conjugation of Benzoic and Salicylic Acids with Glycine

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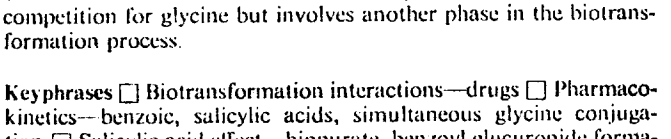
Abstract ☐ The major route of biotransformation of benzoic acid and salicylic acid in man is conjugation with glycine, resulting in the formation of hippuric acid and salicyluric acid, respectively. Both processes are capacity-limited in the usual dose range and approach a maximum rate following administration of sufficiently high doses of precursor. The rate of hippuric acid formation after oral administration of 5 g. benzoic acid in solution is increased markedly by the concomitant administration of glycine. In contrast, the administration of glycine has no effect on the formation of salicyluric acid. Salicylic acid in doses of 1 to 3 g. given orally 2 or 3 hr. prior to 2.0 to 5.0 g. benzoic acid had no measurable effect on the formation of hippuric acid. However, benzoic acid has a pronounced inhibitory effect on the formation of salicyluric acid from salicylic acid. This effect was not prevented by the co-administration of glycine. These results indicate that in man the availability of glycine is rate-limiting in the formation of hippuric acid, but not in the formation of salicyluric acid. Apparently, the inhibitory effect of benzoic acid on the formation of salicyluric acid is not due to competition for glycine but involves another phase in the biotransformation process.

Keyphrases ☐ Biotransformation interactions—drugs ☐ Pharmacokinetics—benzoic, salicylic acids, simultaneous glycine conjugation ☐ Salicylic acid effect—hippurate, benzoyl glucuronide formation, benzoic acid ☐ Benzoic acid effect—salicyluric acid formation, salicylic acid ☐ Glycine effect—benzoic, salicylic acid elimination ☐ UV spectrophotometry—analysis

The elimination of salicylate in man is due mainly to its conjugation with glycine, *i.e.*, the formation of salicyluric acid (1). Since man has a limited capacity for salicylurate formation, the time necessary to eliminate a given fraction of a dose of salicylate increases with increasing dose except in the very low dose range (1, 2). These pharmacokinetic characteristics of salicylate cause this drug to be readily accumulated in the body so that chronic administration may result in intoxication, particularly in young children (3). Treatment of salicylate intoxication is based largely on measures which accelerate the elimination of this drug from the body. It is evident, therefore, that a better understanding of the mechanism and pharmacokinetics of salicylurate formation may lead to safer salicylate therapy and to more effective treatment of salicylate intoxications.

The elimination of benzoic acid in man is due almost exclusively to the conjugation of this drug with glycine, resulting in the formation of hippuric acid (4). This involves the following mechanism:



[1] Adenosine triphosphate.
[2] Coenzyme A.

Presumably, the same sequence of reactions are involved in the formation of salicylurate from salicylate. There are, however, some significant differences in the formation of hippurate and salicylurate. The maximum formation rate of hippurate in man is about 20 times higher than the maximum formation rate of salicylurate (1). The formation of hippurate from benzoate is rate limited by the availability of glycine when benzoate levels exceed about 2 g. in healthy adult man so that administration of glycine with benzoate increases the formation rate of hippurate (5). On the other hand, administration of glycine does not increase the maximum formation rate of salicylurate in man (6). A study of the mutual effects of benzoate and salicylate on the formation of their respective glycine conjugates should be helpful, therefore, in elucidating the rate-limiting step in the formation of salicylurate. This report deals with studies in man; the results of parallel studies in rats will be reported subsequently (7).

THEORETICAL

The formation of hippurate from small doses of benzoate is extremely rapid in man. According to Wu and Elliott (8), the average apparent first-order rate constant for hippurate formation is 10.5 hr.$^{-1}$ while the average apparent first-order rate constant for hippurate excretion is 2.7 hr.$^{-1}$. On the other hand, the formation of hippurate in man occurs at an essentially constant rate for an appreciable length of time after administration of relatively large doses ($\geq$5 g.) of benzoic acid (5, 9). Since, as will be shown subsequently, the renal excretion of hippurate is not rate limited by the capacity of the renal tubular transport system even at the highest excretion rates obtained after administration of benzoate, the following pharmacokinetic model may be applied:

$$\text{benzoate in body} \xrightarrow{R} \text{hippurate in body} \xrightarrow{k} \text{hippurate in urine}$$

where R is the essentially constant rate of hippurate formation and k is the apparent first-order rate constant for hippurate elimination. Since there is no further metabolism of hippuric acid, the rate constant for hippurate elimination (k) is equivalent to the rate constant for hippurate excretion (k_h). The kinetics are similar to those of constant infusion (10), where

$$C = \frac{R}{Vk}(1 - e^{-kt}) \qquad \text{(Eq. 1a)}$$

or

$$A = \frac{R}{k}(1 - e^{-kt}) \qquad \text{(Eq. 1b)}$$

In these equations, C is the concentration of drug (in this case, hippurate) in the blood, V is the apparent volume of distribution of the drug, t is time, and A is the amount of drug in the body. The term $1 - e^{-kt}$ approaches unity when t becomes large. Equation 1*b* then reduces to

$$A = \frac{R}{k} \qquad \text{(Eq. 2)}$$

which shows that the amount of drug in the body attains a constant

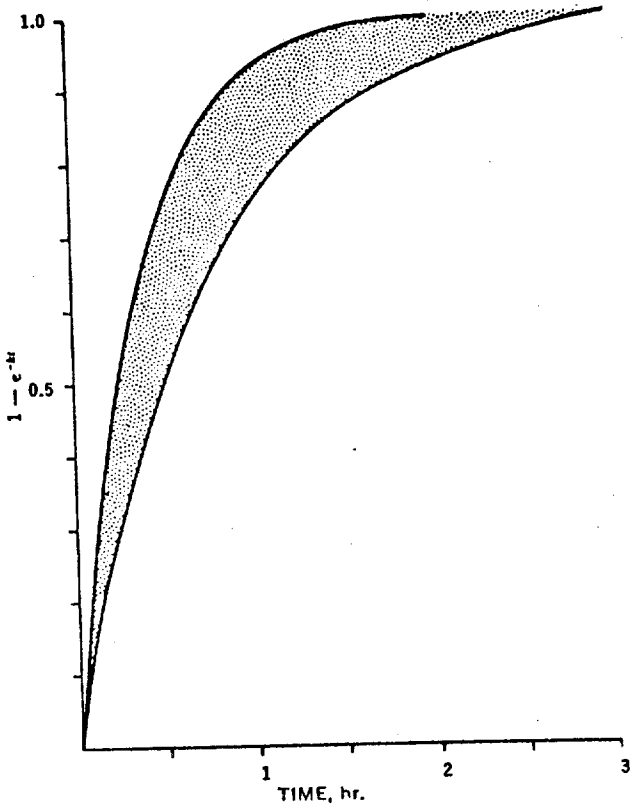


Figure 1—*Plot of 1-e^{-kt} as a function of time. Values for* k *of 2.7 and 1.4 hr.$^{-1}$ were used to construct the upper and lower curves, respectively.*

level independent of time. Since, in the case of hippurate elimination, k is equivalent to the hippurate excretion rate constant k_h

$$\text{excretion rate of hippurate} = kA \qquad \text{(Eq. 3)}$$

Therefore, by substituting R/k from Eq. 2 in Eq. 3,

$$\text{excretion rate of hippurate} \simeq R \ . \qquad \text{(Eq. 4)}$$

when e^{-kt} approaches zero. This indicates that the rate of formation of hippurate can be estimated from urinary excretion data some time after benzoate administration.

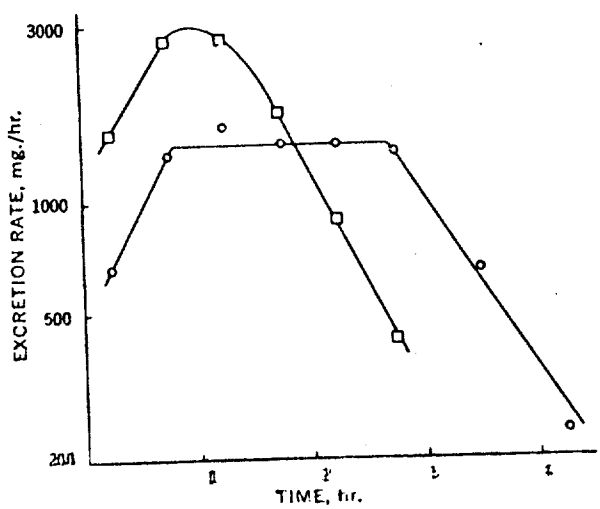


Figure 2—*Effect of glycine (5 g. at −1 hr. and 2 g. every hour thereafter) on the elimination of benzoic acid (Subject A). Shown are the urinary excretion rates of hippuric acid (in terms of benzoic acid) as a function of time after oral administration of 5 g. benzoic acid alone* (O), *and with glycine* (□).

Figure 1 shows theoretical curves for $1 - e^{-kt}$ based on a range of k values from 1.4 to 2.7 hr.$^{-1}$. The high value of k was taken from Wu and Elliott (8); the low value was found in the present study. It is evident that $1 - e^{-kt} \simeq 0.9$ in 1.5 hr. on the average. From that time on, almost constant rates of hippurate excretion should be observed as long as hippurate formation proceeds at an essentially constant rate. During this time, the rate of hippurate excretion will reflect the rate of formation of this metabolite (Eq. 4). An inhibition of the hippurate formation process should therefore be readily apparent from the urinary excretion data. In addition to a lower rate of hippurate excretion, there should occur an increase in the fraction of benzoate which is converted to benzoyl glucuronide. The latter accounts for less than 1% of a dose in the 1 to 2-g. dose range but increases with dose when the dose of benzoate exceeds 2 g. (9).

EXPERIMENTAL

Three healthy male ambulatory human subjects received 2.0 to 5.0 g. benzoic acid as sodium benzoate in aqueous solution. In some of the tests, benzoate was administered 2 hr. after oral administration of 1 to 3 g. salicylic acid as the sodium salt in aqueous solution. The experiments were initiated in the morning after an overnight fast and food was withheld for at least 2 or 3 hr. after benzoate administration. Urine was collected every 30 min. for the first 4 hr., then at longer intervals for a total of at least 8 hr. From 50 to 100 ml. water was ingested after each urine collection to assure adequate urine output. Urine samples were stored in a refrigerator and were usually assayed within 1 day. In experiments with glycine, 5 g. were taken orally in aqueous solution 1 hr. before drug administration and 2 g. every hour thereafter. The experiments involving the administration of hippuric acid were carried out in the same manner as the benzoate studies.

Determination of Benzoate and Hippurate in the Urine Benzoic acid in the urine was determined by acidifying 2 ml. urine with 1 ml. 6 *N* hydrochloric acid and extracting with 30 ml. reagent grade carbon tetrachloride. Ten milliliters of the organic phase was then extracted with 10 ml. of 5% sodium bicarbonate solution. To 5 ml. of the latter was added 1 ml. of concentrated hydrochloric acid. This solution was shaken in a test tube until bubbles of carbon dioxide disappeared, and the absorbance of the solution was then determined at 232 mμ. The absorbance values were corrected for blanks obtained by carrying a sample of water through the same procedure. No measurable hippuric acid is extracted by this procedure. Concentrations of benzoic acid in the presence of salicylic

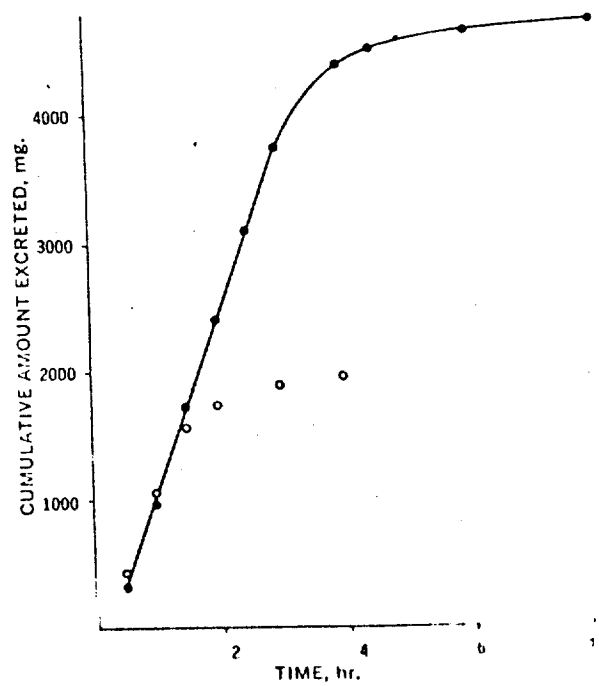


Figure 3—*Cumulative urinary excretion of hippuric acid (in terms of benzoic acid) as a function of time after oral administration of 2 g.* (O) *and 5 g.* (●) *benzoic acid (Subject A).*

Table I—Effect of Dose and Co-administration of Glycine on Benzoic Acid Elimination in Subject A

Dose, g.	Percent of Dose Excreted as Hippuric Acid	Percent of Dose Excreted as Benzoyl Glucuronide	Maximum Excretion Rate of Hippuric Acid, mg./hr.	Hippuric Acid Excretion Rate Constant, hr.$^{-1}$
2.0	95	1.8	1,730	1.2
5.0	94	3.4	2,090	1.1
5.0[a]	100	3.2	2,100	1.2
5.0 + glycine[b]	100	0.6	4,050	1.4
5.0 + glycine[b] + 1.5 g. salicylic acid	98	0.5	3,710	1.4

[a] This experiment was carried out 16 months after the other experiment with the same dose of benzoic acid. [b] See *Experimental*.

acid were determined by two component spectrophotometry (11), at 232 and 305 mμ.

For the determination of hippuric acid, 5 ml. of suitably diluted urine and 5 ml. of concentrated hydrochloric acid were placed in a 20-ml. capacity glass ampul which was sealed and placed in an oven at 100° for 16 hr. A 2-ml. aliquot of this solution was then assayed for benzoic acid as described above. The concentration of hippuric acid in the sample was obtained by subtracting the separately determined concentrations of free benzoic acid and benzoyl glucuronide (expressed as benzoic acid) from the concentration of total benzoic acid. This yielded the hippuric acid concentration in terms of benzoic acid.

Determination of Benzoyl Glucuronide—Five milliliters of urine (diluted with water if necessary) and 2 ml. of 0.4 *M* acetate buffer, pH 4.5 were placed in each of two 25-ml. capacity glass-stoppered flasks. One milliliter β-glucuronidase[3] was added to one flask and 1 ml. water to the other. The solutions were then incubated at 37° for 16 hr. Two milliliters of the solution was acidified with 1 ml. 6 *N* hydrochloric acid and extracted immediately into carbon tetrachloride. The assay then proceeded as described above for benzoic acid. The results thus obtained represent both free benzoic acid and benzoyl glucuronide; subtraction of the separately determined free benzoic acid concentration yields the concentration of benzoyl glucuronide.

Recoveries and Blank Values—The recoveries of hippuric acid and benzoic acid in solutions containing various known concentrations of these two substances as well as salicylic acid were generally between 98 to 100%. Apparent hippuric acid output in nonmedicated subjects ranged from 15 to 30 mg. benzoic acid equivalent/hr., which is in the range of normal values (12). The apparent excretion rate of benzoic acid in nonmedicated subjects was less than 0.4 mg./hr.

Determination of Salicylate and Metabolites—Salicylate and its metabolites were determined in the urine as previously described (13).

RESULTS

Formation of Hippurate from Benzoate—Figure 2 shows the urinary excretion rate of hippurate as a function of time after oral administration of 5 g. benzoate. Hippurate was excreted at an essentially constant rate of about 2.1 g./hr. (equivalent to 1.4 g. benzoic acid/hr.), from about 1 to about 3 hr. after benzoate administration. When benzoate was administered together with glycine (5 g. at −1 hr. and 2 g. every hour thereafter), the rate of hippurate excretion was increased to 4.1 g./hr. (equivalent to 2.8 g. benzoic acid/hr.). However, the slope of the terminal exponential excretion phase of hippurate was the same in each instance.

Figure 3 is a plot of cumulative amount of hippurate excreted as a function of time after oral administration of 2 and 5 g. benzoate, respectively. It is apparent that the initial excretion rate of hippurate following the lower dose is quite similar to that after the higher dose of benzoate. Benzoate was almost quantitatively recovered in the urine as hippurate, except for a minor fraction which was excreted as benzoyl glucuronide (Table I). No free benzoic acid was detected in the urine; the sensitivity of the analytical method used in this study was sufficient to detect 0.5% benzoyl glucuronide of a total dose of 2 g. benzoic acid under the experimental conditions. The

[3] Ketodase containing 5,000 units/ml., Warner-Chilcott, Morris Plains, N. J.

Table II—Elimination Kinetics of Hippuric Acid

Subject	Dose, g.	Percent Recovered in Urine	Maximum Excretion Rate, mg./hr.	Hippuric Acid Excretion Rate Constant, hr.$^{-1}$
A	2.9	92	1,380	1.1
A	5.0	106	2,030	(0.74)[a]
A	7.5	96	2,600	(0.92)[a]
B	2.9	92	1,790	1.3

[a] Questionable value, probably affected by prolonged absorption phase.

2-g. dose of benzoate yielded relatively less benzoyl glucuronide than the 5-g. doses; concomitant administration of glycine with 5-g. benzoic acid resulted in a decrease in benzoyl glucuronide output (Table I).

The excretion rate constant of hippuric acid was essentially the same when this metabolite was formed in the body from benzoate or when it was administered as such (Tables I and II). The maximum excretion rate of hippurate increased with increasing oral dose of this metabolite, but this relationship was not linear (Table II).

Effect of Salicylate on the Formation of Hippurate from Benzoate—Administration of 2 g. salicylic acid 2 hr. before 2 g. benzoic acid had no apparent effect on the elimination of the latter (Fig. 4). A higher dose of salicylate (3 g.) also did not affect hippurate output (Fig. 5). Furthermore, salicylate did not prevent the enhancement of hippurate formation by exogenous glycine (Fig. 6). Salicylate had no measurable effect on the proportion of hippuric acid and benzoyl glucuronide excreted in the urine after benzoate administration, and on the excretion rate constant of hippuric acid (Fig. 5, Tables III and IV).

Effect of Benzoate on Salicyluric Acid Formation from Salicylate—

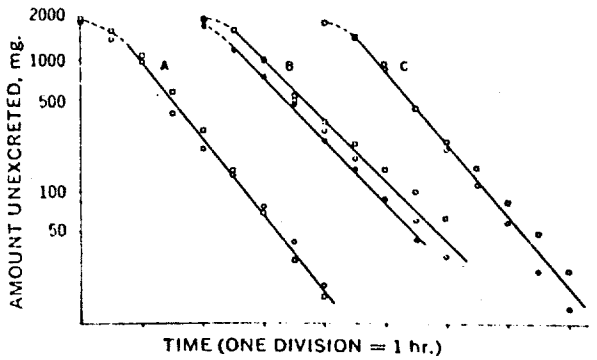


Figure 4—*Effect of salicylic acid on the elimination of benzoic acid in three human subjects. Plotted are the total amounts of benzoate metabolites (mainly hippuric acid) remaining unexcreted as a function of time after oral administration of 2 g. benzoic acid alone* (○), *and 2 hr. after oral administration of 2 g. salicylic acid* (△). (●), *amount of hippurate unexcreted following oral administration of 2.9 g. hippuric acid (equivalent to 2 g. benzoic acid). Amounts unexcreted are expressed in terms of benzoic acid.*

Table III—Effect of Salicylic Acid (SA) on Elimination of Benzoic Acid (BA)

Subject[a]	Dose of BA, g.	Percent of Dose Excreted as Hippuric Acid		Percent of Dose Excreted as Benzoyl Glucuronide		Hippuric Acid Excretion Rate Constant, hr.$^{-1}$	
		Control	With SA	Control	With SA	Control	With SA
A(M,25,90)	2.0	95	100[c]	1.8	1.3	1.2	1.4
B(M,24,84)	2.0	105	102[c]	0.4	0.8	1.2	1.0
C(M,34,72)	2.0	95	94[c]	2.5	1.0	1.3	1.0
A	5.0 and glycine[b]	100	98[d]	0.6	0.5	1.4	1.4

[a] In parentheses: sex, age in years, body weight in kilograms. [b] See *Experimental*. [c] 2.0 g. SA at −2 hr. [d] 1.5 g. SA at −3 hr.

Figure 7 depicts the time course of salicyluric acid excretion in Subject A after oral administration of 2 g. salicylate when 2 g. benzoate was given 2 hr. later. Similar results were obtained in Subjects B and C.[4] The salicyluric acid excretion rate reached a plateau at about 1 hr. and decreased precipitously immediately after benzoate administration. It then increased again and returned to the plateau level at about 5 hr. It is noteworthy that the excretion rate of salicyluric acid at 6 to 8 hr. was essentially the same as at 1 hr. even though the amount of salicylate in the body had decreased by more then one third. Co-administration of a large amount of glycine did not prevent the inhibition of salicyluric acid formation by 2 g. benzoate (Fig. 8). Figure 9 shows the excretion rate of salicylurate as a function of time after administration of salicylate and a larger dose (3.2 g.) of benzoate with and without glycine. Glycine reduced, but did not prevent, the inhibitory effect of benzoate on salicylurate formation.

DISCUSSION

Capacity-Limited Elimination of Benzoate—The data in Table I and in Figs. 2 and 3 show that the formation of hippurate from benzoate is capacity limited, the availability of glycine being the rate limiting factor. Consistent with this is the increase with increasing dose in the fraction of benzoate excreted as the glucuronide, and the decrease in this fraction when glycine is administered together with benzoate (Table I). Even with as low a dose as 2 g. of benzoic acid, hippurate formation is approaching its maximum rate (Fig. 3, Table I). Following an oral dose of 5 g. benzoic acid, hippurate excretion reached its maximum level somewhat earlier than predicted by theory (Fig. 1). This was observed also in other experiments and is probably due to the presence of some endogenous glycine at the site of biotransformation. Only after this initial "pool" of glycine is depleted somewhat will the rate of supply of additional glycine become the limiting factor in the synthesis of hippurate. The maximum rate of hippurate formation, as reflected by the urinary excretion data, is similar to that observed by other workers (reviewed in *Reference 1*). Since the results of the benzoate elimination experiments agreed with and confirmed the results and conclusions of other investigators, this aspect of the investigation was not pursued further.

Hippurate Elimination Kinetics—The rate constant for hippurate excretion ranged from 1.1 to 1.4 hr.$^{-1}$ regardless if the metabolite was formed in the body or administered as such (Tables I–IV). Salicylate had no measurable effect on hippurate excretion in the dose range studied (Table IV). Wu and Elliott (8) observed a considerably higher average hippurate excretion rate constant (2.7 hr.$^{-1}$) in 25 subjects, while Hirsheimer (14), in a study of 58 subjects obtained data following intravenous administration of hippurate which yielded an average value of 1.6 hr.$^{-1}$, in good agreement with the results of the present study. The maximum excretion rate of hippurate did not increase linearly with increasing dose (Table II). This may have been due to partial precipitation of hippuric acid

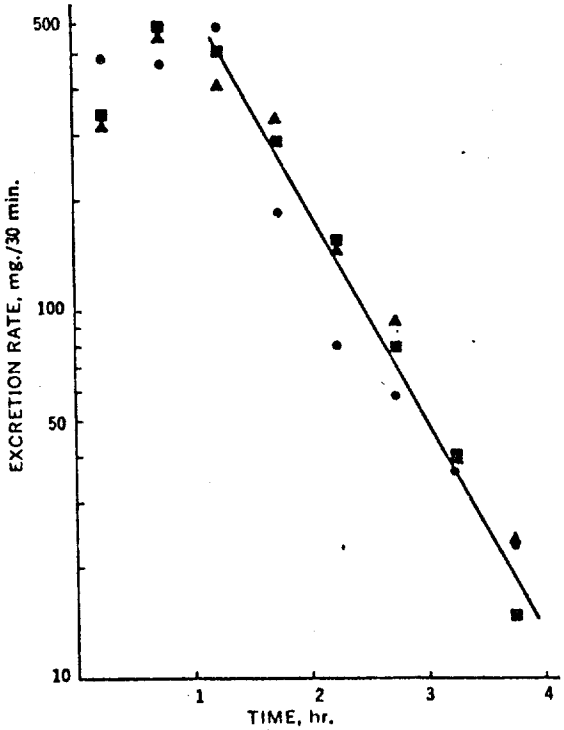


Figure 5—*Effect of different doses of salicylic acid on the elimination of benzoic acid. Shown are the urinary excretion rates of hippuric acid (expressed in terms of benzoic acid) after oral administration of 2 g. benzoic acid alone (●), 2 hours following 2 g. salicylic acid (■), and 2 hours following 3 g. salicylic acid (▲). Subject A.*

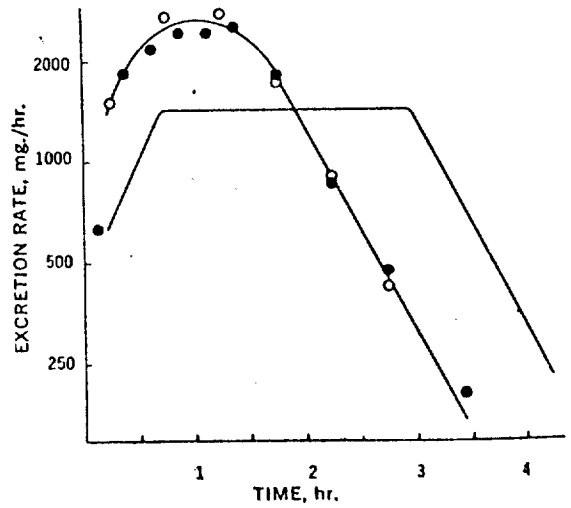


Figure 6—*Effect of salicylic acid on the availability of exogenous glycine for hippurate formation (Subject A). Excretion rates of hippurate (in terms of benzoic acid) as a function of time after oral administration of 5 g. benzoic acid and glycine (5 g. at −4 hr. and 2 g. every hour thereafter) with (●), and without (○), salicylic acid (1.5 g. at −3 hr.). The lower curve is taken from Fig. 2 and represents the urinary excretion rates of hippurate after administration of 5 g. benzoic acid without glycine in the same subject.*

[4] These data are available from the authors upon request.

Table IV—Effect of Different Doses of Salicylic Acid on Elimination of Benzoic Acid in Subject A

Dose, g. BA	Dose, g. SA	Percent Recovered in Urine	Percent of Dose Excreted as Hippuric Acid	Benzoyl Glucuronide	Hippuric Acid Excretion Rate Constant, hr.$^{-1}$
2.0	—	97	95	1.8	1.2
2.0	1.0	96	94	1.3	1.3
2.0	2.0	101	100	1.3	1.4
2.0	2.0 + glycine[a]	91	89	1.1	1.2
2.0	3.0	99	98	1.4	1.1

[a] See *Experimental.*

in the stomach and/or delayed gastric emptying,[5] resulting in relatively slower absorption of the large doses.

Effect of Salicylate on Benzoate Elimination—Administration of 2 g. salicylic acid, a dose at which salicylurate formation is capacity limited and proceeding at an essentially constant and maximum rate (1), had no measurable effect on hippurate formation (Fig. 4). Consistent with this is the fact that benzoyl-glucuronide formation, a parallel and competing process for benzoate elimination, did not increase during salicylate administration (Table III). Even when the dose of salicylate was increased to 3 g., there was no apparent inhibition of hippurate formation. It is particularly interesting that salicylate also does not interfere with the availability of exogenous glycine for hippurate formation (Fig. 6), in view of the fact that there exists a metabolic inhomogeneity of glycine in man and that plasma glycine is used preferentially for hippurate synthesis (15). These results are contrary to those of Little *et al.* (16) who found that salicylate inhibits the formation of *para*-aminohippurate in liver and/or kidney slices of rats, dogs, and rabbits. On the other hand, it has been stated by Cummings *et al.* (17) that salicylate does not affect the formation of *para*-aminohippurate from *para*-aminobenzoate in man, although no experimental data were presented in support of this statement.

Effect of Benzoate on Salicylurate Formation—Benzoate had a rapid and pronounced inhibitory effect on salicylurate formation (Fig. 7). The duration of this effect was short, consistent with the relatively rapid elimination of benzoate. It has been shown previously that repeated administration of small doses of benzoate (0.5 g. every 30 min.) can inhibit salicylurate formation for extended periods of time (18). The magnitude of the inhibitory effect was dose-dependent (Fig. 7 *versus* Fig. 9). The essentially constant excretion rate of salicylurate before and after benzoate, in a period of time when the amount of salicylate in the body decreased by nearly 50%, reflects the capacity-limited nature of the salicylurate formation process (1, 19). Concomitant administration of large amounts of glycine did not prevent the inhibitory effect of benzoate on salicylurate formation (Figs. 8 and 9), but reduced the magnitude and duration of inhibition. This is readily apparent in Fig. 9 and is due to the more rapid elimination of benzoate when glycine is given. Glycine did not increase the formation rate of salicylurate before benzoate administration or after benzoate elimination. This is consistent with the results of previous studies in this laboratory (6). It has also been shown previously that benzoate has no apparent effect on salicylurate excretion (18).

Rate-Limiting Factors in Hippurate and Salicylurate Formation, and Mechanism of Interaction Between Benzoate and Salicylate—The results of this and previous studies (5) show that the availability of glycine is rate limiting in the formation of hippurate, but that this is not the rate-limiting factor in the formation of salicylurate. It is evident also (Figs. 4, 5, and 9) that salicylate does not inhibit the access of endogenous or exogenous glycine to the site of hippurate formation. The apparent lack of effect of salicylate on hippurate formation may be due to a greater affinity of benzoate to the enzyme responsible for the conjugation with glycine; it could also reflect a greater relative distribution of benzoate, as compared to salicylate, to the site of biotransformation. On the other hand, the formation of salicylurate and hippurate may involve different enzyme systems, similar to what has been found with respect to glucuronide formation (20, 21). Thus, Schachter and Taggart have found that the activating enzyme involved in the formation of benzoyl CoA does not form salicyl-CoA (22). However, attempts to obtain salicylurate synthesis even in liver and kidney slices (16) or homogenates (23) have not been entirely successful. Until salicylurate formation can be consistently demonstrated *in vitro*, the results of Schachter and Taggart cannot be taken as definite proof of

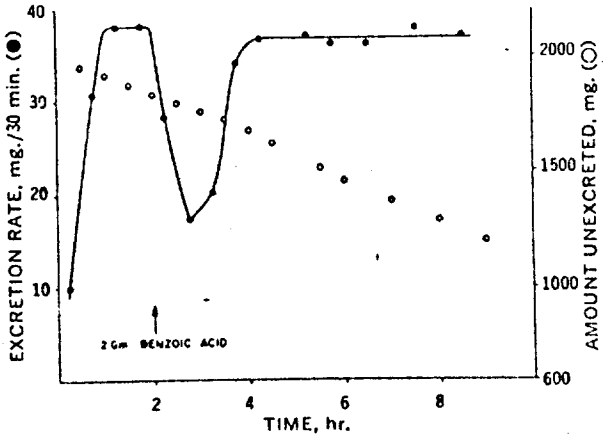


Figure 7—*Effect of benzoic acid on the formation of salicyluric acid from salicylic acid by Subject A. Shown are the urinary excretion rates of salicyluric acid (●) and the amounts of salicylate remaining in the body (○) as a function of time after oral administration of 2 g. salicylic acid. Two grams benzoic acid was given 2 hr. after administration of salicylic acid.*

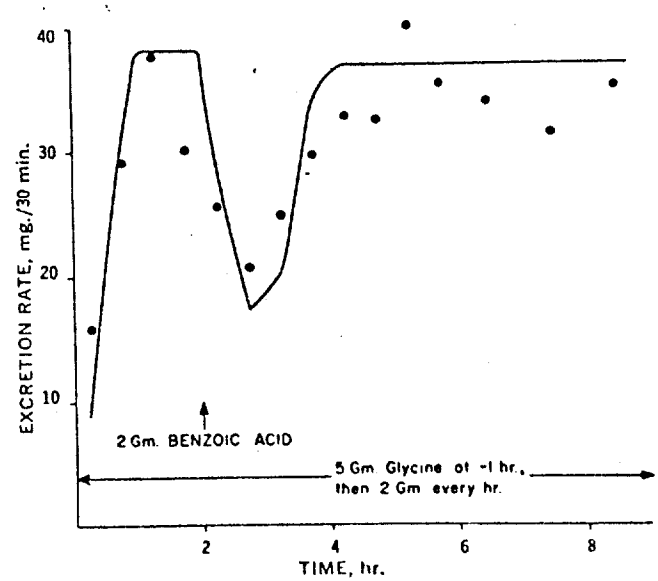


Figure 8—*Effect of benzoic acid on the formation of salicyluric acid from salicylic acid during co-administration of glycine (Subject A). Shown are the urinary excretion rates of salicyluric acid as a function of time after oral administration of 2 g. salicylic acid. The curve is taken from Fig. 6 and represents the excretion rates observed when glycine was not given.*

[5] The larger doses caused some nausea, which in turn retards gastric emptying (unpublished observations).

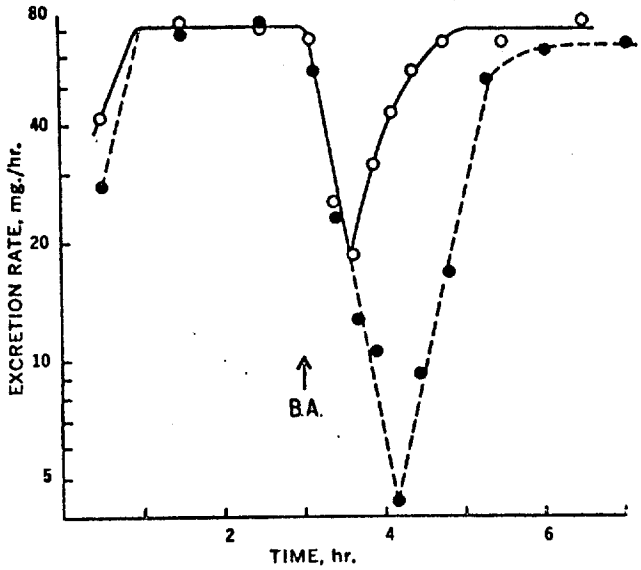


Figure 9—*Effect of benzoic acid on the formation of salicyluric acid from salicylic acid with and without administration of glycine (Subject A). Shown are the urinary excretion rates of salicyluric acid as a function of time after oral administration of 1 g. salicylic acid and 3.2 g. benzoic acid 3 hr. later, with* (O), *and without* (●), *glycine.*

the existence of an enzyme capable of activating benzoate, but not salicylate. If, however, there are more than one substrate-activating and/or glycine-transferring (*N*-acylase) enzymes with different substrate specificity, it may also be that one substrate inhibits the glycine conjugation of another substrate, but not the reverse. This is known to occur in glucuronide conjugation (20), where there is increasing evidence for the existence of two or more enzyme systems with different substrate specificity (20, 21). The results of the present study suggest that the same may be the case with respect to glycine conjugation.

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ACKNOWLEDGMENTS AND ADDRESSES

Received August 23, 1968, from the *Biopharmaceutics Laboratory, Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214*

Accepted for publication November 15, 1968.

Presented to the Pharmacology and Biochemistry Section, APhA Academy of Pharmaceutical Sciences, Miami Beach meeting, May 1968.

Supported in part by grant No. 1R01 DS00021-01 from the U. S. Public Health Service, Bethesda, Md., and by a fellowship for L.P.A. from the American Foundation for Pharmaceutical Education.

Previous paper: G. Levy and J. A. Procknal, *J. Pharm. Sci.*, **57**, 1330(1968).

Journal of Medicinal and Pharmaceutical Chemistry
Vol. 2, No. 6 (1960)

Potential Anticancer Agents*—L. Non-Classical Antimetabolites—II. Some Factors in the Design of Exo-Alkylating Enzyme Inhibitors, particularly of Lactic Dehydrogenase

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Introduction

Among the earliest known biochemical differences between normal and neoplastic tissues were the lowered oxygen uptake and high lactate formation of some neoplastic tissues compared to most normal tissues.[1] The enzymatic shunt of pyruvate to lactate with resultant regeneration of DPN‡ from DPNH is probably necessary for oxidative anabolism in an oxygen-deficient cell. Since this function of LDH is probably unessential for most, if not all, normal tissues, then a strong selective blockade of this enzyme could result in selective inhibition of a tumour that is dependent on this reaction for regeneration of DPN.

The observation by Ottolenghi and Denstedt[2] that phenoxyacetic acid is an inhibitor of LDH, in either of the directions that the enzymatic reaction is run, suggested that this moiety, and related rather large molecules (compared to pyruvate), be investigated further in cancer chemotherapy. The fact that phenoxyacetic acid and a variety of its common derivatives failed to inhibit

* This programme is carried out under the auspices of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Public Health Service, Contract No. SA 43 ph 4892. The opinions expressed in this paper are those of the authors and are not necessarily those of the Cancer Chemotherapy National Service Center. For the preceding paper in this series, *cf.* W. A. Skinner, M. G. M. Schelstraete, and B. R. Baker, *J. org. Chem.*, **26** (1961). In press. For paper I on non-classical antimetabolites, see reference 4.

‡ The following abbreviations are used: DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; LDH, lactic dehydrogenase; GDH, glutamic dehydrogenase.

the growth of Sarcoma 180, Adenocarcinoma 755 or Leukemia L-1210* might be explained by the high *in vivo* concentration of pyruvate with which these inhibitors must compete, and the relatively poor inhibitions[2] of LDH-catalyzed pyruvate reduction by phenoxyacetic acid.† An inhibitor that could combine irreversibly with LDH should be much more effective,[4] since one inhibitor molecule could combine with one active site on LDH and selectively denature the enzyme, rather than competing with substrate pyruvate.‡

There are two known types of irreversible inhibitors:[4] those that bind ionically to the enzyme much more strongly than to the

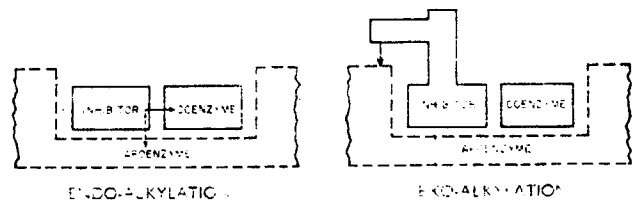


Fig. 1. Oversimplified diagram of endo- and exo-alkylation. The arrows represent the attack of an alkylating group on an active site. The two different routes for endo-alkylation are shown by the solid arrow and by the dotted arrow.

substrate (pseudo-irreversible[6, 7]) and those that combine chemically with the enzyme through covalent bond formation to stop desorption. The latter can be divided into two classes:[4] those that react by endo-alkylation such as azaserine, and those that could theoretically react by exo-alkylation (Fig. 1).

In order to design an effective exo-alkylating irreversible inhibitor of LDH, the following factors should be considered:

(1) Relatively large moieties (compared to pyruvate), other than phenoxyacetic acid, that could serve as a carrier for the alkylating group giving exo-alkylation of the enzyme;

* Personal communication from Dr. Howard W. Bond, Cancer Chemotherapy National Service Center; *cf.* also reference 3.

† These negative results could also be interpreted to mean that the phenoxyacetic acids are not selective enough in their inhibitor action, or even that the pyruvate to lactate shunt is of no value to a tumour cell.

‡ Busch *et al.*[5] have recently observed that dichloropyruvate strongly inhibited the growth of Walker rat Carcinosarcoma 256 and attributed the drug action to irreversible inhibition of LDH.

(2) The size, position, and inductive effect of substituents on the phenoxyacetic acid and other moieties that can be made without losing inhibitory action;

(3) Determination of the effect of the inhibitors in (1) and (2) on an enzyme with similar binding sites, such as GDH, in order to obtain an approximate estimate of the comparative specificity;

(4) The placing of a suitable alkylating group on an inhibitor, such as phenoxyacetic acid, that has sufficient chemical reactivity and the proper dimensions to be able to alkylate a nucleophilic group of the enzyme, such as NH, OH, or SH, near the active site (see Fig. 1).

Factors (1), (2) and (3) are the experimental subjects of this paper and factor (4) the future projection of this work.

Experimental

Enzyme Measurements

Reagents

DL-Lactate, L-glutamate, and DPN were commercial preparations. Crystalline LDH, isolated from rabbit muscle, and crystalline GDH, isolated from mammalian liver, were purchased from Nutritional Biochemicals Corporation. The sources of inhibitors are indicated in the Tables.

Methods of Assay

The enzymatic activities of LDH[2] and GDH were measured by the rate of change of concentration of DPNH. The concentration of DPNH was determined by the change in optical density of the medium at 340 mμ with either a Beckman DU spectrophotometer or a Cary 14 recording spectrophotometer.

In the reverse reaction (lactate → pyruvate), the reaction mixture contained 0·05 molar phosphate buffer (pH 8·4), DL-lactate at a final concentration of 8 mmolar, DPN at a final concentration of 2·4 mmolar, and, when used, an inhibitor. All the ingredients, except the enzyme, were placed in a cuvette with a total volume of 3·0 ml. The reaction was started by adding the proper quantity of LDH in 0·10 ml of the phosphate buffer. Sufficient enzyme was used to give a rate of formation of DPNH of about 0·4 optical density units per minute when the above

concentrations of DL-lactate and DPN were employed. It was observed that D- and L-lactate gave the same rate of reaction; therefore the convenient DL-lactate was employed.

The GDH assays were run similarly[8] in 0·05 molar phosphate buffer (pH 8·4) with a final concentration of 2 mmolar L-glutamate, 0·8 mmolar DPN, and sufficient enzyme to give a rate of formation of DPNH of about 0·25 optical density units per minute.

The forward reaction (pyruvate → lactate) was run similarly in 0·05 molar tris buffer (pH 7·4) with a final concentration of 2 mmolar sodium pyruvate, about 0·6 mmolar DPNH, and sufficient enzyme to give a rate of change of about 0·6 absorbence units per minute.

Chemistry

Commercial materials, if not pure, were recrystallized until their melting points agreed with accepted literature values.

3-Ethoxyphenoxyacetic acid (Method A). To a refluxing solution of *m*-ethoxyphenol (1·38 g, 10 mmoles) in 4 per cent aqueous sodium hydroxide (10 ml, 10 mmoles) was added dropwise over 30 min a solution of chloroacetic acid (0·95 g, 10 mmoles) in 2 per cent aqueous sodium hydroxide (20 ml, 10 mmoles). The solution, after being refluxed for 1–2 h, was acidified to pH 1 and cooled in an ice-bath. The product was collected on a filter and washed with water; yield, 1·01 g (52 per cent), m.p. 89–90°.

Recrystallization from benzene–petroleum ether (b.p. 30–60°) gave colourless crystals, m.p. 89–89·5°.

Anal. Calcd. for $C_{10}H_{12}O_4$: C, 61·2; H, 6·12. Found: C, 61·3; H, 6·33.

In cases where the phenol was not sufficiently soluble and precipitated with the substituted phenoxyacetic acid, the crude product was dissolved in ethyl acetate and the phenoxyacetic acid extracted out with 5 per cent aqueous sodium bicarbonate. Acidification gave the phenoxyacetic acid, usually in pure form. Recrystallization was employed when necessary.

The 3-phenoxypropionic acids were prepared in the same manner using 3-bromopropionic acid.

3-Aminophenoxyacetic acid hydrochloride (Method B). A mixture of *m*-acetamidophenoxyacetic acid (1·0 g, 4·8 mmoles) (Table I) and 12 N hydrochloric acid (15 ml) was refluxed for 2 h,

then chilled in an ice-bath. The product was collected on a filter and washed with ice-cold 6 N hydrochloric acid: yield, 0·70 g (72 per cent), m.p. > 300°. The compound gave a single spot (R_f 0·75) when chromatographed on acetylated paper in benzene-methanol-water (2 : 6 : 1).

Anal. Calcd. for $C_8H_9NO_3.HCl$: C, 47·2; H, 4·90; Cl, 17·4. Found: C, 46·9; H, 4·91; Cl, 17·4.

3-(2-Aminophenoxy)propionic acid (Method C). A mixture of 3-(2-nitrophenoxy)propionic acid[9] (1·00 g, 4·72 mmoles), absolute ethanol (50 ml), and 5 per cent palladium–charcoal (75 mg) was shaken with hydrogen for 15 min, when reduction was complete. The filtered solution was evaporated to dryness *in vacuo*, leaving 0·70 g (82 per cent) of pure product, m.p. 106–107°.

Anal. Calcd. for $C_9H_{11}NO_3$: C, 59·6; H, 6·07. Found: C, 59·3; H, 5·97.

Results

A plot of V_0/V against I for two or more concentrations of the inhibitor was used to determine the concentration of I necessary to give 50 per cent inhibition ($V_0/V = 2$) in the presence of 8 mmolar lactate, where V_0 = velocity of reaction with no inhibitor, V = velocity with inhibitor, and I = concentration of inhibitor.[10] Only those concentrations of inhibitor giving 30–70 per cent inhibition were employed for the plots in order to avoid the high errors in higher or lower inhibitions. With most of the inhibitors, a straight line was obtained and the point of 50 per cent inhibition was readily estimated using a few points only. Some obviously non-competitive* inhibitors gave a bowed curve. In these cases more points were obtained, with one near 50 per cent inhibition, so that the 50 per cent inhibition point could be determined with reasonable accuracy; the obvious non-competitive inhibitors are marked in the Tables. The 50 per cent inhibition concentrations were then divided by the concentration of substrate to give the I_{50} values, which are defined as the millimolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 millimolar concentration of substrate. The I_{50} values for GDH

* Throughout this paper, non-competitive inhibition is defined as any type of inhibition that is not competitive, rather than the strict kinetic definition of non-competitive,[10] unless otherwise indicated.

and the LDH forward reactions were determined similarly; the I_{50} values are recorded in the Tables. This definition of I_{50} allows a more direct comparison of the effect of the same inhibitor on two different enzymes such as GDH and LDH, although kinetically speaking one cannot reduce the 50 per cent inhibition values at a given concentration of substrate to the I_{50} value at 1 millimolar substrate concentration unless the inhibitor is competitive. Nevertheless, if one compares the experimentally observed 50 per cent inhibition values for oxidation by GDH and LDH, one comes to the same general conclusions set forth in the Discussion.

Discussion

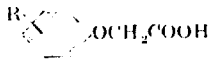
Rationale

The purpose of the present investigation was to find relatively large moieties that could inhibit LDH and would be suitable for later conversion to exo-alkylating inhibitors.[4] With this objective in mind, we felt that the examination of a wide variety of compounds for inhibition was more important than the achievement of maximal quantitative accuracy for any particular inhibitor.*

Attempts to compare the I_{50} values of competitive *vs.* non-competitive inhibitors may be grossly inaccurate and unsatisfying from the enzymologist's strictly kinetic standpoint. Nevertheless, the chemotherapist should not ignore attempts to make these comparisons, since an invading intact cell may be killed as selectively by blockade of an enzyme essential to this cell by a non-competitive antagonist with poor kinetics as by a competitive antagonist with good kinetics. The major objection to non-competitive antagonists—the commonly believed statement[23] that 'it may be anticipated that competitive antagonists will exhibit greater specificity than non-competitive'—is subject to severe criticism in the light of some of the results given in Tables III and IV. For example, Hellerman *et al.*[8] have done careful kinetic studies showing that isophthalate and glutarate are equally effective ($I_{50} = 1$) competitive antagonists of GDH.

* This philosophy of approach for a broad survey of an enzymatic phenomenon has been previously expressed by Levy and Vennesland.[22]

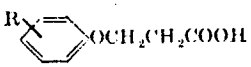
Table I. Inhibition of LDH (reverse reaction) and GDH by substituted phenoxyacetic acids



R	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	m.p., °C	Literature m.p., °C	Method or source[b]
H	3·0	41	13			D
2-CH_3	6·3	28	4·4	155–156	151–152[11]	A
3-CH_3	6·6	28	4·3	104–105	102[11]	A
4-CH_3	14	28	2·0	140–141	135[11]	A
3,5-$(CH_3)_2$	4·1	109	27	110–111	110–111[12]	A
2-Cl	2·4	11	4·6			E
3-Cl	3·2	13	4·1	109–109·5	108–110[13]	A
4-Cl	3·4	11	3·2			F
2,4-Cl_2	1·6	3·5	2·2			E
2,4,5-Cl_3	0·67	2·5	3·7			D
2,4,5-Cl_3-6-CH_3	1·0	2·0	2·0			D
2,4,6-Cl_3	1·5	10	6·7			G
4-HO	22	45	2·3			E
3-CH_3O	4·5	34	7·6	117·5–118	118[14]	A
4-CH_3O	6·7	35	5·1			E
3-C_2H_5O	6·1	17	2·8	89–90		A[c]
4-C_2H_5O	4·2	26	6·1			E
3-n-C_3H_7O	2·1	3·5	1·5	98–98·5[d]		A[d]
4-NH_2	12	39	3·2	200–210(d.)	200–210(d.)[15]	C[e]
3-NH_2[f]	4·0	89	22	>300		B[c]
2,4-Cl_2-5-NH_2	1·4	6·5	4·7			H
3-AcNH	8·3	80	9·6	170–171	171–172[16]	A
2-AcNH	5·5	50	9·1	149–149·5	153–154[17]	A
3,4-Benzo	0·93	2·0	2·1			J

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] For Methods A, B and C, *cf.* Experimental; D, Matheson, Coleman and Bell; E, Eastman Kodak Co.; F, California Foundation for Biochemical Research; G, K. and K. Laboratories; H, Dr. Howard W. Bond, Cancer Chemotherapy National Service Center; J, Berkeley Chemical Corporation. [c] See Experimental. [d] *Anal.* Calcd. for $C_{12}H_{16}O_3$: C, 64·3; H, 7·14. Found: C, 64·4; H, 7·18. [e] Product precipitated during hydrogenation. The mixture was filtered through a Celite pad. The latter was continuously extracted in a Soxhlet apparatus with ethanol to recover the product. [f] As the hydrochloride; the free base had been prepared previously.[15]

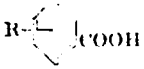
Table II. Inhibition of LDH (reverse reaction) and GDH by substituted 3-phenoxypropionic acids



R	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	m.p., °C	Method or source[b]
H	3·9	15	3·8	94–95[c]	A
3-AcNH	4·0	37	9·2	132–134[d]	A
2-NH_2	6·3	72	11	106–107[e]	C

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] For Methods A and C, *cf.* Experimental. [c] Lit. m.p. 96–97°.[18] [d] *Anal.* Calcd. for $C_{11}H_{13}NO_4$: C, 54·7; H, 6·23. Found: C, 54·4; H, 6·48. [e] See Experimental.

Table III. Inhibition of LDH (reverse reaction) and GDH by substituted benzoic acids



R	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	Source[b]
H	3·4	50[c]	15	A
2-HO	0·75	20	27	B
3-HO	0·87	14[c]	16	A
2-COOH	34	126[c]	3·7	B
3-COOH	6·1	1·0[d]	0·16	C
2,4-$(COOH)_2$	35	30	0·86	D[e]
3,5-$(COOH)_2$	34	3·0[f,g]	0·088	A
3-COOH-5-CH_3	7·4	40	5·4	D[e]
3-COOH-5-AcNH	2·3	30	13	D[e]
3-COOH-4-AcNH	1·9	30	16	D[e]
3-COOH-5-OH	1·9[h]	2·5	2·3	A
3,4-Benzo	1·8	4·0	2·2	A
2,3-Benzo	2·4[i]	10	4·2	A
3,4-Benzo-2-HO	0·062	0·10[h]	1·6	A
3,4-Benzo-6-HO	0·19	0·70[h]	3·7	A

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] A, Eastman Kodak Co.; B, J. T. Baker Co.; C, Matheson, Coleman and Bell, D, synthetic. [c] Uvetic acid, m.p. 270–280°; synthesized in two steps from pyruvic acid.[19] [d] Prepared from 5-aminoisophthalic acid by acetylation.[20] [e] Prepared from 4-aminoisophthalic acid by acetylation.[21] [f] Previously studied by Hellerman *et al.*[8] but data determined in this laboratory. [g] m.p. 220–224 (d.), prepared by hydrolysis of anhydride obtained from Amoco Chemical Corporation. [h] Strongly non-competitive. [i] Moderately non-competitive.

Table IV. Inhibition of LDH (reverse reaction) and GDH by miscellaneous compounds

Compound	LDH[a] I_{50}	GDH[a] I_{50}	GDH I_{50} / LDH I_{50}	Source[b]
Oxalate	0·21	100	480	A
Glutarate	82	0·60[c]	0·0073	A
Methoxyacetate	> 100	192	< 1·9	A
Ethoxyacetate	23	238	10	A
Phenylmercaptoacetate	4·7	25	5·3	E
DL-Mandelate	8·3	80	9·6	A
Phenylpyruvate	2·7	36	13	B
3-Benzoylpropionate	4·6	48	10	C
Oxanilate	2·2	19	8·6	A
Cinnamate	3·1	33[c]	11	A
Hydrocinnamate	> 100	45	< 0·45	A
Furoate	6·4	18[c]	2·8	A
5-Bromofuroate	3·3	0·050[c]	0·015	D

[a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] A, Eastman Kodak Co.; B, Nutritional Biochemical Corporation; C, K. and K. Laboratories; D, Dr. Howard W. Bond, Cancer Chemotherapy National Service Center; E, Evans Chemetics, Inc. [c] Previously studied by Hellerman *et al.*[7]

Table V. Inhibition of LDH forward reaction (pyruvate → lactate)

Compound	LDH[a] I_{50}	GDH[a, b] I_{50}	GDH I_{50} / LDH I_{50}
Oxalate	0·50	100	200
Oxamate[c]	0·30	9·0	30
Oxanilate[d]	14	19	1·4
Phenoxyacetate	70	41	0·58
	35[e]	41	1·2
Benzoate	240	50	0·21
m-Hydroxybenzoate	48	14	0·29
	22[e]	14	0·63
Salicylate	19	20	1·1
	17[e]	20	1·2
5-Bromosalicylate[c]	5·5[e]	1·8	0·33
5-Chlorosalicylate[c]	5·5[e]	2·5	0·45
Phenylpyruvate	21	36	1·7
Levulinate[d]	> 500[e]		
Cinnamate	33	33	1·0
Isophthalate	71	1·0	0·014
5-Bromofuroate	104	0·050	0·00048

[a] Sources are not indicated, see previous Tables. [a] mmolar concentration of inhibitor required to give 50 per cent inhibition in the presence of 1 mmolar concentration of substrate. [b] Glutamate + α-oxoglutarate. [c] Matheson, Coleman and Bell Co. [d] Eastman Kodak Co. [e] Non-competitive. [f] pH 8·4, 0·05 M tris buffer. [g] This result does not agree with that recorded by Busch and Nair.[28]

However, glutarate (Table IV) has little effect on the LDH reverse reaction except at high concentrations ($I_{50} = 82$), whereas isophthalate (Table III) is a reasonably good inhibitor ($I_{50} = 6 \cdot 1$) of the LDH reverse reaction. Thus two equally effective competitive inhibitors for GDH vary by a factor of 22 on specificity of inhibition for LDH; therefore, the fact that an inhibitor is competitive for a given enzyme affords no assurance that the inhibitor is selective.

On the other hand, trimesic acid (5-carboxyisophthalate, Table III) is a good non-competitive inhibitor of GDH ($I_{50} = 3 \cdot 0$) but is still more effective on GDH than on LDH ($I_{50} = 34$); thus, a non-competitive inhibitor may be more selective than a competitive inhibitor. Admittedly, any one of the compounds in the tables may be a more effective inhibitor for some other enzyme in an intact cell; nevertheless, conclusions on specificity drawn from comparison of two enzyme systems have infinitely more validity than any invalid conclusions on specificity drawn from measurements on only one enzyme.

Ideally, one should measure the effect of an inhibitor on all the enzymes present in a cell to understand the specificity. Since this is obviously impractical, chemotherapy has developed mainly on the basis of the effect of an inhibitor on an intact organism, the selectivity of the effect being measured as the chemotherapeutic index. In the final analysis, the drug is useful only if it has an acceptable chemotherapeutic index in the intact organism. Many thousands of references can be quoted, including some from this laboratory,[24, 25] where structure activity relations have been attacked by a purely empirical approach, albeit successfully. However, the empirical approach to chemotherapy and the design approach attempted in this manuscript are not mutually exclusive; in fact, there is considerable crossover between the two approaches—intentional or not. Thus the possible validity or non-validity of certain concepts in chemotherapy can be supported at the enzyme level by comparison of the effects of inhibitors on two enzyme systems such as GDH and LDH, as discussed in the following sections.

Chemotherapeutic Implications

The efficacy of a drug is best measured by its chemotherapeutic index, that is, the ratio of the maximum tolerated dose to the

minimum effective dose; these are usually expressed as 50 per cent values for obvious experimental reasons. Then the ED_{50} is the dosage regimen that will be effective in 50 per cent of the host organisms. LD_{50} is the dose given with the same regimen that causes 50 per cent lethality, and the chemotherapeutic index, C.I., is LD_{50}/ED_{50}. Thus, the ED_{50} is a measure of effect of the drug on the desired enzyme (or receptor site), whereas the LD_{50} is a measure of the effect on the most critical, but undesired, enzyme or receptor site of the host. If one reduces this to its simplest terms and ignores the less lethal side-effects of the drug for the moment, then one can make an analogy with any two enzyme systems.

For the sake of discussion, let us assume that the LDH reverse reaction is the target enzyme that must be inhibited for the desired physiological effect on a tumour; then the I_{50} of an inhibitor of this reaction can be likened to the ED_{50}. Similarly, if GDH is assumed to be a critical enzyme for the host, inhibition of which will lead to lethality, then the I_{50} of the inhibitor on GDH can be likened to the LD_{50}.

In structure–activity studies, one varies the active structure empirically, then measures the chemotherapeutic index in the given host system.[24,25] The most effective change, that is, the change giving the best chemotherapeutic index, makes the compound more effective against the target receptor site than the lethal receptor site.

Among the substituted phenoxyacetic acids in Table I, phenoxyacetic acid has $I_{50} = 3 \cdot 0$ against LDH and $I_{50} = 41$ against GDH. Thus the ratio of the I_{50}'s, namely 13, would be the chemotherapeutic index for this system. In chemotherapy, the compound active at the lowest dose in a series is not necessarily the best compound. For example, (2,4,5-trichlorophenoxy)acetic acid is the most active compound in Table I with an I_{50} of $0 \cdot 67$, but its selectivity (chemotherapeutic index) is decreased to $3 \cdot 7$. In contrast, (3-aminophenoxy)acetic acid is less active ($I_{50} = 4 \cdot 0$) than the parent phenoxyacetic acid, but has greater selectivity with an inhibition ratio of 22.

In Table III, benzoic acid ($I_{50} = 3 \cdot 4$) is almost as good an inhibitor of the LDH reverse reaction as phenoxyacetic acid. This activity is greatly enhanced by substitution of a hydroxyl

group on position 2 ($I_{50} = 0 \cdot 75$) or 3 ($I_{50} = 0 \cdot 87$) of the ring. Of these two compounds, salicylic acid has the better selective effect with a ratio of 27. The corresponding naphthalene analogue, 1-hydroxy-2-naphthoic acid, is twelve times more effective ($I_{50} = 0 \cdot 062$) than salicylic acid in inhibiting the LDH reverse reaction: in fact, it is the most active compound that we have observed in this system. However, it is far from the most selective compound, since its inhibition ratio compared to GDH is only $1 \cdot 6$.

The fact that almost all the compounds in Tables I–V do inhibit both enzymes at some level might be interpreted to mean that these compounds are general enzyme poisons and would affect many other enzymes. Actually, as will be discussed later, there is a fair degree of similarity between the binding sites on LDH and GDH and this was one of the reasons GDH was selected for comparison purposes. To show that even a highly effective but less selective inhibitor, such as (2,4,5-trichlorophenoxy)acetic acid, is not a general enzyme poison, a dehydrogenase enzyme system requiring a phosphate instead of a carboxylate for enzyme binding of the substrate was investigated. Thus, (2,4,5-trichlorophenoxy)acetic acid was totally ineffective as an inhibitor of glucose 6-phosphate dehydrogenase.*

It can be anticipated—or at least hoped—that basic knowledge in enzymology and chemotherapy will eventually reach the point where the chemotherapist will be able to compare the isolated target enzyme with the isolated enzyme in which the undesirable side-effect can occur. That such an approach could be useful in the future can be seen from the data on substituted isophthalates (Table III). If the target enzyme were lactic dehydrogenase, it is clear that isophthalate would not be detected as a possible inhibitor when tested in the intact organism, since its fair ($I_{50} = 6 \cdot 4$) inhibition of LDH would be masked by its stronger ($I_{50} = 1 \cdot 0$) inhibition of GDH. In contrast, with the isolated enzyme systems, it is possible to use isophthalate as a screening lead for structure–activity studies. Note that the bulk of a 5-methyl group on isophthalate makes little change ($I_{50} = 7 \cdot 4$) on the inhibition of LDH, but causes a 40-fold decrease ($I_{50} = 40$)

* We wish to thank Drs. W. Kilgore and J. Greenberg of these laboratories for this determination.

in inhibition of GDH, which is very sensitive to third-dimensional bulk.[8] Thus an unfavourable inhibition ratio of 0·16 that could not be detected in the intact organism has been changed to an inhibition ratio of 5·4, which could be a sufficiently favourable chemotherapeutic index in the intact organism to allow detection of its activity. Furthermore, the 4-acetamido group on isophthalate gives a compound more active ($I_{50} = 1 \cdot 9$) than phenoxyacetate ($I_{50} = 3 \cdot 0$) and with the good inhibition index of 16.

The fate of pyruvate in such a comparative study would be of obvious use in the design of LDH inhibitors for cancer chemotherapy since: (1) pyruvate reduction by LDH is an enzymic reaction probably essential for some types of cancer cells, but of no use for most normal cells, and (2) oxidation of pyruvate to acetyl coenzyme A with pyruvic dehydrogenase is a reaction essential for energy generation in all cells. Such a study should be made, since little is known about inhibition of pyruvic dehydrogenase; any inhibitor which would be as effective on pyruvic dehydrogenase as on LDH would be of no utility, as discussed below.

Busch and Nair[26] have stated the case for inhibitors of LDH as potential anticancer agents. They re-emphasized that inhibitors of the LDH oxidation of lactate do not necessarily inhibit the LDH reduction of pyruvate. In addition, they succinctly stated that 'Inasmuch as inhibition of glycolysis in tumours would require inhibition of the forward reaction, all the experiments in the present studies were carried out with DPNH and pyruvate as substrates. It was found that fluoropyruvate exhibited a more marked suppression of the reduction of pyruvate to lactate than the other available inhibitors.' Unfortunately, fluoropyruvate was 'too perfect' as a classical type antimetabolite. It not only inhibited the reduction of pyruvate* but also inhibited the oxidation of pyruvate to acetate by pyruvic dehydrogenase,[26] a reaction essential for normal cells; as a result, the compound was highly toxic and showed no inhibition of the mouse tumours Sarcoma 180, Adenocarcinoma 755, or Leukemia L 1210,† or

* Of more than passing interest is the fact that fluoropyruvate was observed to be an irreversible inhibitor of LDH,[26] most probably of the 'enzyme-alkylating' type.

† Private communication from Dr. Howard W. Bond, Cancer Chemotherapy National Service Center.

of the Walker rat Carcinosarcoma 256[5] at the maximum tolerated dose.

Classical antimetabolite theory, as expounded by Woolley[27] and Roblin,[28] greatly limits the change in the structure of a metabolite that should be made to convert it to an antimetabolite; that is, the antimetabolite should be as close in structure to the corresponding metabolite as possible. In a small molecule such as pyruvate, about the only small change that classical antimetabolite theory allows to be made in the structure is substitution of a fluorine for a hydrogen, since the remainder of the molecule is necessary for binding to the enzyme.[29] As already pointed out, Busch and Nair[26] have made this change, but the resultant fluoropyruvate suffered from lack of specificity. Thus, classical antimetabolite theory rules out any further chance of designing a selective inhibitor of LDH reduction of pyruvate.

In contrast, non-classical antimetabolite theory[4] states that 'an antimetabolite should be as close as possible in structure to that part of the molecule where the stereospecific requirements of the enzyme surface must be met.'

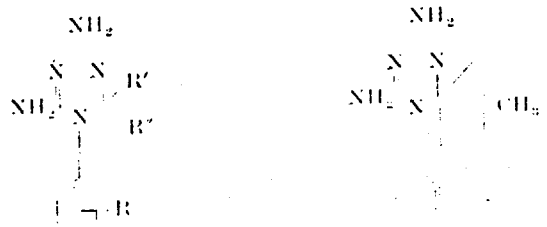
Furthermore, the conclusions reached earlier in this paper with non-classical inhibitors such as 4-acetamidoisophthalate and phenoxyacetate make it possible to propose a second corollary of non-classical antimetabolite theory. This corollary, a direct antithesis of classical theory, can be stated as follows:

In order for an antimetabolite to have maximum enzyme specificity, the greatest possible changes in the bulk of the antimetabolite should be made that still allow the stereospecific and binding requirements of the target enzyme to be met.

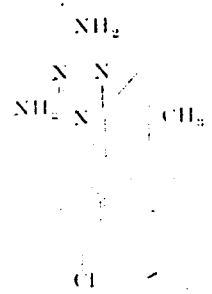
Available evidence for this corollary has been known for twenty years; in fact, this evidence appeared soon after the original Woods Fildes antimetabolite theory[30,31] was proposed. The discovery of the first useful antibacterial agent, sulphanilamide, led directly to the antimetabolite theory when a few years later *p*-aminobenzoic acid was predicted and found[30] to be the competitive substrate. As a result of classical structure activity relationship studies,[32] sulphanilamide was soon replaced by other sulphonamides, such as sulphathiazole, sulphadiazine, etc. in clinical practice since they exhibited fewer side reactions. Note that the heterocyclic rings of these drugs represent a bulky

change in structure compared to sulphanilamide, the classical antimetabolite of *p*-aminobenzoic acid. Thus, they represent an early example of non-classical antimetabolites that have been modified with greater bulk to give more specificity to the inhibitor.

One of the most recent examples of proper bulk for enzyme specificity has been observed in the folic reductase area.[33] The 4,6-diamino-1-aryl-1,2-dihydro-*sym*-triazines (I) have been known to be folic reductase inhibitors for some years,[34] are useful for the treatment of malaria,[34,35] and show some anticancer activity,[34] particularly when R′ and R″ are small groups such as methyl



(I)



(II)

and R is a *meta* or *para* group such as halogen. The anticancer and antimalarial activity, as well as toxicity, were lost when R was an *ortho* group or R′ and R″ became bulky. In contrast, pinworms such as *Syphacia obvelata* in mice were very susceptible to the diaminotriazines (I), if R was a bulky group in the *o*-position or R′ and R″ were bulky groups. The compound of choice,[33] 1-(*p*-chlorophenyl)-4,6-diamino-1,2-dihydro-2,2-(3′-methylpentamethylene)-*sym*-triazine (II) had high anthelmintic activity in mice and exceedingly low toxicity. There is little doubt that the phenyl group of (II) is restricted in its rotation and cannot assume a planar conformation with the triazine ring. Even though Roth *et al.*[33] state that compounds such as (II) no longer have any significant antifolic activity—presumably when assayed by the usual microbiological techniques such as *Lactobacillus casei*, *L. arabinosus*, or *Streptococcus faecalis*—it is logical to assume that

the folic reductase enzyme of *Syphacia obvelata* can still be blocked by (II) in its nonplanar conformation, whereas the folic reductase of mammals, *L. arabinosus*, *L. casei*, or *S. faecalis*, can only accept a relatively planar biaryl conformation. The reality of this proposition could best be verified by inhibitor studies on folic reductase prepared from cell extracts of *Syphacia obvelata*. That the same enzyme can differ in inhibitor specificity from species to species,[36] from 'wild type' cells to resistant cells,[37] or even from tissue to tissue of the same animal,[38] has been observed previously.

Thus, in order to design an effective inhibitor, it would be most useful if data were available on the requirements and limitations of the active site on the enzyme of the species and tissue in question.* Ideally, this study should employ LDH obtained from the tumour tissue for which inhibition is desired, preferably a human tumour. Since this ideal cannot be met at this time for obvious technical reasons, this pilot study was initiated with commercial LDH isolated from rabbit muscle so that a suitable experimental approach could be found for determining the nature of the enzyme site.

Nature of the Active Site on LDH

The need for and use of knowledge of the stereospecificity, binding, and allowance for bulk at the active site on LDH were discussed in the previous section. As Busch and Nair[26] point out, it is necessary to block the reduction of pyruvate catalyzed by LDH and not the oxidation of lactate in order for an LDH inhibitor to be an effective anticancer agent. Nevertheless, considerable useful information about the enzyme site can be obtained by study of the inhibition of lactate oxidation that cannot be as readily obtained by inhibition of the forward reaction. The fact that a variety of inhibitors that compete with the reverse reaction are not inhibitors of the forward reaction[26] can be, and has been,[39]

* The proposition that the same enzyme from different tissues of the mouse differs in its ability to be inhibited by the 6-aminonicotinamide analogue of DPN[38] and that the triazine (II) presumably also differs in its ability to inhibit the same enzyme from different organisms, suggests that additional studies should be made on key anabolic enzymes to see if there are exploitable differences in enzyme specificity between a variety of normal tissues and different types of tumour tissues.

explained by invoking different sites for pyruvate and lactate on LDH. This concept has been refuted by the elegant work of Schwert *et al.*[40] on LDH. They state:

"In our model this hypothesis is not necessary. The location of the binding site for lactate and for pyruvate is presumably the same as regards the geometry of the enzyme surface and in part in the bound coenzyme. Since DPN and DPNH differ in both steric and chemical properties, the configuration of the binding site for substrate or inhibitors on the enzyme DPN complex is quite different from that on the enzyme DPNH complex."

Further studies by Winer and Schwert[29] on the quenching of

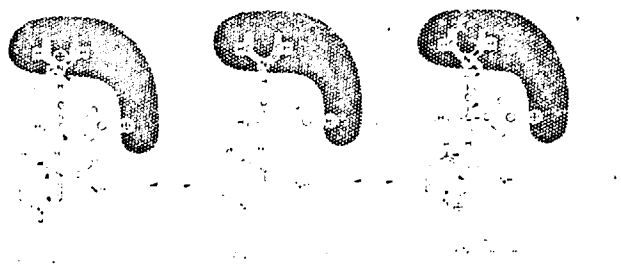


Fig. 2. Modified Winer-Schwert two-dimensional model for LDH. The shaded area represents protein surface. Note that the activated complex at the transition state is identical.

the fluorescence spectra of the LDH-DPNH complex by the classical-type antimetabolite, oxamate, caused them to propose a two-dimensional working model of the LDH-DPNH-pyruvate and the LDH-DPN-lactate complexes, a slight modification of which is presented in Fig. 2. Our present studies with non-classical inhibitors of LDH agree with the type of transition state proposed by Winer and Schwert. A three-dimensional model would be more desirable, since it more closely approximates the true nature of the enzyme site; our experiments were designed for this purpose.

The Dennis-Kaplan[11] three-dimensional model for oxidation of lactate, when applied to non-classical inhibitors (Fig. 3), fails to show why phenoxyacetate with an α-CH_2 group is a good inhibitor of the oxidation of lactate (Table I) but a poor inhibitor of the reduction of pyruvate (Table V). In Fig. 3, where the DPN is

in the imidazole plane, the equatorial hydrogen of DPNH[22] is in about the same position as the 4-hydrogen of DPN. Thus, the DPN and DPNH, when placed in this position, do not modify the geometry of the enzyme appreciably. Molecular models* show that phenoxyacetate can fit either the LDH-DPNH complex or the LDH-DPN complex equally well. Experimentally, this is not the case with phenoxyacetate. The model in Fig. 3 also does not account for the logical assumption of Levy and Vennesland[22] that the attack of pyruvate by the hydride of DPNH axial to the plane of the DPNH would involve the smallest shift of atoms from the positions which they occupy in the planar DPN form.

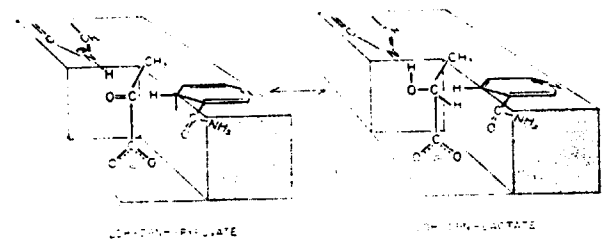


Fig. 3. Modified Dennis-Kaplan three-dimensional model for LDH.

The Levy-Vennesland hypothesis is satisfied if the DPN and DPNH are in a plane perpendicular to the plane of the imidazole ring on the protein surface and parallel to the line between the carboxyl carbon and the α-carbon of the substrate (Fig. 4). In this configuration, the equatorial hydrogen of the bound DPNH (axial with respect to DPNH) in its LDH complex constitutes a change in the enzyme geometry compared to the DPN-LDH complex. Construction of molecular models clearly showed that the phenoxyacetate would readily fit the enzyme site for LDH-DPN; in contrast, phenoxyacetate could bind only poorly to the LDH-DPNH site *because of the interaction of an α-hydrogen of phenoxyacetate with the equatorial hydrogen of the DPNH.* This equatorial hydrogen plus the slight extension of the 4-carbon of the pyridine ring in DPNH from the vertical plane in the model (Fig. 4), due to the boat form[22] of the pyridine ring, most prob-

* Catalin Molecular Models manufactured by Catalin Products, Ltd., Waltham Abbey, Essex, England.

ably account for the difference in the specificity between LDH–DPN and LDH–DPNH.

Non-classical antimetabolites did fit the LDH–DPNH complex provided the carbon next to the carboxyl binding had a double-bonded oxygen rather than a $-CH_2-$ group. Thus, oxanilate was five times as good an inhibitor ($I_{50} = 14$) (Table V) and phenylpyruvate ($I_{50} = 21$) more than three times as good an inhibitor as phenoxyacetate ($I_{50} = 70$). However, in the model in Fig. 4, there was considerable restricted rotation of the benzene ring in both oxanilate and phenylpyruvate, which can account for part of the poorer I_{50} demonstrated by these compounds on reduc-

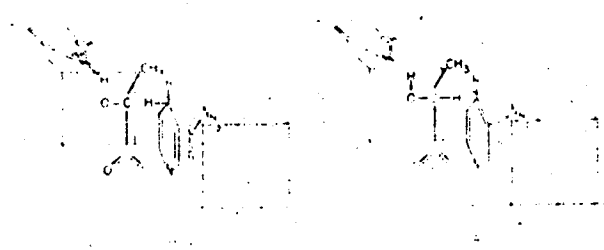


Fig. 4. Three-dimensional model of LDH satisfying the current inhibition studies.

tion of pyruvate compared to oxidation of lactate; an important factor that makes the LDH–DPNH complex more difficult to inhibit than the LDH–DPN complex is that pyruvate binds about 10^3 times more strongly to the LDH–DPNH complex than lactate binds to the LDH–DPN complex.[32] It should be possible to overcome this difficulty with an exo-alkylating irreversible inhibitor.[1] The fact that oxanilate and phenylpyruvate are still reasonably good inhibitors shows that outside the range of interference by the equatorial hydrogen of DPNH at the exo-face of the inhibitor, gross changes in structure can be made.

A less obvious molecule, salicylate (Table V) is also a better non-classical inhibitor ($I_{50} = 19$) of the LDH–DPNH complex than phenoxyacetate. The salicylate inhibition of LDH–DPNH can be increased by halogen substitution on the 5 position of salicylate ($I_{50} = 5{\cdot}5$) although specificity is decreased; it could most likely be increased by proper positioning of bulk, as proposed earlier.

Salicylate ($I_{50} = 0{\cdot}75$) and its isomer, 3-hydroxybenzoate ($I_{50} = 0{\cdot}87$), gave useful information on the height of the plane of the imidazole ring, since both compounds are excellent and almost equally effective inhibitors of the LDH–DPN complex.* The height of the imidazole plane must be just above the hydroxyl of salicylate when its carboxyl is bound to the enzyme, but just below the hydroxyl of 3-hydroxybenzoate when its carboxyl is bound to the enzyme. This height is still satisfactory for the LDH–DPNH–pyruvate and LDH–DPN–lactate complexes, as shown in Fig. 4.

The substituted phenoxyacetic acids, when measured as inhibitors of the oxidation of lactate by DPN (Table I), gave considerable further information on the nature of the active site of LDH. Phenoxyacetic acid should have its primary binding to the LDH–DPN complex (Fig. 4) through the carboxylate group as usual. Secondary binding of a hydrogen-bond type can then occur between the ether oxygen of phenoxyacetate[2] and the NH of the imidazole; note that it must be assumed that the imidazole ring is protonated for this binding to the LDH–DPN complex. The ether oxygen fits just below the imidazole NH in a fashion similar to the LDH–DPNH–pyruvate complex (Fig. 4). Molecular models then show that there is still room for the phenyl group, but the adjacent 2- and 6-hydrogens cause some restricted rotation of the phenyl ring; this restricted rotation is increased only slightly by 2,6-dichloro-substituents, but the fit is still good and the inductive effect of the halogens (discussed later) more than overcomes the increased restriction of rotation.

* Among the assumptions made in the classical derivation of the mathematical expressions for enzyme inhibition[46] are: (1) only competitive inhibitors can fit the active site of an enzyme, (2) non-competitive inhibitors combine with some site other than the active enzyme site (ESI complex), and (3) the rate-limiting step in an enzymic reaction is the decomposition of the enzyme–substrate complex to regenerate enzyme and products. Some workers, including ourselves, disagree that these assumptions are invariably valid.[47,48] In fact, it is possible to make other assumptions, not invoking an ESI complex, to explain the kinetics of non-competitive inhibition;[49] thus, non-competitive inhibitors can, and probably do, involve the active site. In order to avoid obscuring the more pertinent issues, we determined that salicylate and 3-hydroxybenzoate gave strictly competitive inhibition of LDH–DPN–lactate when plotted by the Lineweaver–Burk method;[50] phenoxyacetate has previously been shown to be competitive.[2] Thus, in the discussion that follows, the active site of LDH must account for the fit of these inhibitors.

The good inhibition of LDH by (3,5-dimethylphenoxy)acetate (I_{50} = 4·4) gives an important clue to the position of the carboxamide group of the DPN bound to LDH. If the relatively planar carboxamide group of DPN is in front as pictured in Fig. 4 (or at the rear) and held by the enzyme on a plane perpendicular to the DPN plane, then molecular models show that there is considerable interaction between a methyl group of (3,5-dimethylphenoxy)-acetate and the carboxamide group of the DPN. This can only be demonstrated by the presence of two *m*-methyl groups, since with only one *m*-methyl group, the phenyl moiety can be rotated 180° to remove the methyl group from the immediate vicinity of the carboxamide group, thus allowing much more freedom of rotation of the phenyl moiety. Since (3,5-dimethylphenoxy)-acetic acid is nearly as good an inhibitor of LDH as phenoxyacetic acid, it is clear that the plane of the carboxamide group of the DPN cannot be perpendicular to the plane of the DPN, but that the carboxamide group must approach coplanarity with the DPN. However, no evidence appears to be available to indicate whether the carboxamide group is at the front (as indicated in Fig. 4) of the DPN or at the rear of the DPN, provided the carboxamide group is coplanar.

That there is open space to the left of the 2′ methylene group of lactate in the LDH–DPN–lactate binding, as shown in Fig. 4, is clearly indicated by the fact that substitution of the bulky methyl group on the 2-position of (2,3,5-trichlorophenoxy)acetate (I_{50} = 0·67) causes little change in binding (I_{50} = 1·0).

The inductive effects of substituents on the binding of phenoxyacetate (Table I) to the LDH–DPN complex deserves notice. In a general way, halogens in the *o*- or *p*-position tend to increase binding (lower I_{50}), in contrast to amino or hydroxyl groups in these positions, which decrease binding. However, a rigid correlation with the *sigma* constant or ionization constant of either the phenoxy acid or its corresponding phenol could not be demonstrated.

Active Site of Glutamic Dehydrogenase

The inhibition of GDH by 5-methyl-, 5-acetamido-, 5-carboxy-, and 5-H isophthalate (Table III), with I_{50} values of 40, 30, 3·0, and 1·0, respectively, lends further credence to the conclusion

of Hellerman *et al.*[8] that the inhibitors of GDH must be planar in their conformation: that is, the active site can tolerate little third-dimensional bulk since the decrease in I_{50} values in this series of substituted isophthalates corresponds to the decrease in third-dimensional bulk as shown by molecular models. Further inhibitor studies would be necessary to establish whether or not third-dimensional bulk could be accepted by the enzyme if this

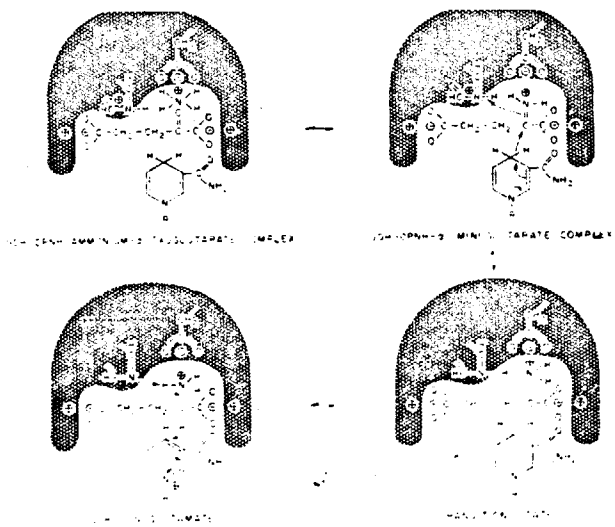


Fig. 5. Two-dimensional model for GDH. The shaded area represents protein surface.

bulk were further removed from the binding groups of the inhibitor.

Sufficient data are available to propose a two-dimensional model of the GDH enzyme site (Fig. 5) that is similar to the two-dimensional model of LDH (Fig. 2). In addition to the imidazole ring on the protein surface for proton transfer, it would also be convenient to invoke the presence of a carboxylate group on the enzyme site with the proper conformation to bind the various forms of ammonia involved, that is, ammonium ion, the imino-glutarate zwitterion, and the glutamate zwitterion. Comparison of the two-dimensional model of LDH (Fig. 2) with that of GDH

(Fig. 5) shows some obvious similarities. Thus, one could expect some inhibitors to inhibit both enzymes. As discussed earlier, a particular inhibitor can be made to be more effective for one enzyme than the other, or *vice versa*, by judicious placing of other substituents.

Attempts to draw a three-dimensional model of GDH are probably not warranted with the data available, since the inhibitors studied were primarily chosen for the information they could give on the LDH active site (Fig. 4).

Conclusion

Further experimental work is warranted on compounds related to oxanilate, phenylpyruvate, and salicylate with proper substituents to give irreversible inhibition of LDH, since the current study has shown that space is available on the LDH site for inhibitors to have the large groups necessary for construction of the theoretically possible exo-alkylating type irreversible inhibitor.[4] Detailed information on inhibition of pyruvate dehydrogenase would be of considerable practical importance, although Bosund[13] has observed that salicylate does not inhibit pyruvic dehydrogenase in resting cells of *Proteus vulgaris*.

Summary. A second corollary of non-classical antimetabolite theory has been proposed, based on comparative inhibition of lactic dehydrogenase and glutamic dehydrogenase by several series of inhibitors. In addition, considerable insight has been gained on the three-dimensional aspects of the active site of lactic dehydrogenase, which could be of considerable use for the design of potential anticancer agents of the irreversible type.

(*Received 6 September, 1960*)

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Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 66 OCTOBER, 1947 No. 1

15962

A Comparison of Effect of Caronamide and Benzoic Acid on Penicillin Plasma Concentrations.

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The excretion of penicillin by the renal tubules can be inhibited by diodrast,[1,2,3] para-aminohippuric acid,[3,4,5] benzoic acid and sodium benzoate,[6,7,8] and caronamide.[9,10,11] Although both diodrast and para-aminohippuric acid have been used clinically to increase penicillin plasma concentrations, the necessity of administering intravenously large quantities of the drugs has limited their general usefulness. Benzoic acid, sodium benzoate and caronamide can be administered orally and may therefore be more widely used in conjunction with penicillin therapy. Since no comparison between the effects of benzoic acid and the new compound, caronamide, has appeared in the literature, such a comparison is here reported.

Plan of Study. Seven individuals who were afebrile and without evidence of cardiac, renal, or hepatic dysfunction, were selected for this study. Each patient was studied during 3 periods and served as his own control. Hospital routine was not modified and no effort was made to limit the intake of food or fluid.

In order that assayable plasma concentrations of penicillin might be anticipated during the entire 3 hour period over which dose response curves were obtained, intramuscular doses of 200,000 units were employed. This dose was dissolved in 2 cc of sterile saline and the sodium salt of penicillin G‡ was used exclusively. The penicillin dose given immediately before the determination of peni-

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"Staticin" caronamide (4'-carboxyphenylmethanesulfonanilide) used in this investigation was supplied through the courtesy of Sharp and Dohme, Inc.

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cillin dose response curves was injected into the right deltoid region by one of the authors and massage of the injection site was avoided.

The pattern of patient study was as follows: *Control day.* An intramuscular injection of 200,000 units of penicillin at 6 and 9 A. M. Following the 9 o'clock injection into the right deltoid region, blood specimens for penicillin assay were obtained at intervals of 15 minutes, 30 minutes, 1 hour, 2 hours and 3 hours (this schedule of sampling is referred to as a dose response curve). *Benzoic acid treatment day.* Each patient was given benzoic acid every 3 hours for 24 hours before a dose response curve was determined. At the same time that the 6 and 9 A. M. doses of benzoic acid were given, an intramuscular injection of 200,000 units of penicillin was administered. Following the 9 o'clock penicillin and benzoic acid medication a dose response curve was obtained. Benzoic acid was administered in 0.5 g gelatin capsules; patients JJ, PR, JC and JA received 2 g every three hours. Patients SB, JG and MV received 3 g every 3 hours. *Caronamide treatment day.* This was similar to the benzoic acid treatment day except that caronamide in 0.5 g tablets was administered in place of benzoic acid.

It should be pointed out that, (a) in all instances at least a three day interval intervened between the benzoic acid and caronamide treatment days; (b) in the case of patients JJ, PR, JC and JA the administration of benzoic acid preceded that of caronamide, whereas patients SB, JG and MV received caronamide before benzoic acid; and (c) patients JJ and SB were incompletely studied so that a caronamide treatment day is lacking for JJ and a benzoic acid treatment day is lacking for SB.

Methods. Blood specimens for penicillin assay were drawn into sterile syringes containing 1 cc of 0.4% sodium citrate solution and were promptly transferred to graduated 15 cc centrifuge tubes. The specimens were centrifuged for 15 minutes at 1500 rpm and the total volume as well as the packed cell volume were recorded. The supernatant plasma was harvested and refrigerated in contact with ice and remained so until penicillin assay on the same or subsequent day. Assays were done by the modified [12] serial dilution method of Rammelkamp using a Group A hemolytic streptococcus as the test organism. All penicillin values recorded in Fig. 1 are corrected for the dilution factor introduced by use of the citrate anticoagulant.

Discussion. It is apparent from the data shown in Fig. 1 that caronamide had a striking effect upon penicillin plasma concentrations when administered orally in doses of 2 or 3 g every 3 hours. Equally apparent is the lack of any significant effect of benzoic acid when given in doses of 2 or 3 g every 3 hours. Six patients showed a marked increase of penicillin plasma concentrations in response to caronamide therapy and 6 patients failed to show any favorable response to benzoic acid treatment. The individual responses to caronamide and to benzoic acid were quite uniform but the averaged data are most striking (Fig. 1). Whereas the benzoic acid dose response curve corresponds exactly to that obtained during the control period, the dose response curve modified by caronamide shows fold increases: at 15 minutes—1.67, 30 minutes—3.57, 1 hr.—3.83, 2 hr.—5.35 and 3 hr.—11.68 (average 5.22) times the control values. The enhancement effects noted in this study are in good agreement with those previously noted. [10, 11]

That benzoic acid had little effect on penicillin plasma concentrations if patients were permitted to have an unrestricted diet was noted by Bronfenbrenner and Favour.[7] These authors sharply restricted fluid and salt intake so that "the urine volume commonly fell to 400 to 600 cc in 24 hours," and gave 2.5 g of benzoic acid in capsules every 4 hours. On this regimen, enhancement effects were demonstrated and were ascribed to benzoic acid.

The patients studied in our series were given food and fluid ad libitum and doses of 16 and 24 g of benzoic acid per day. No elevation of penicillin plasma concentrations

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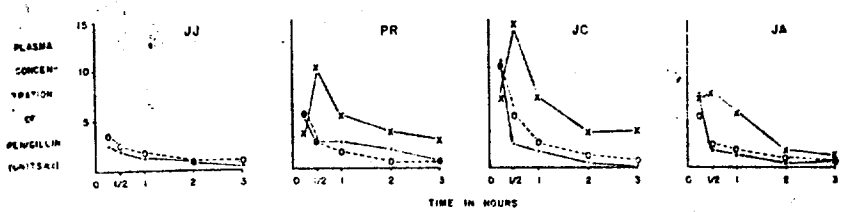
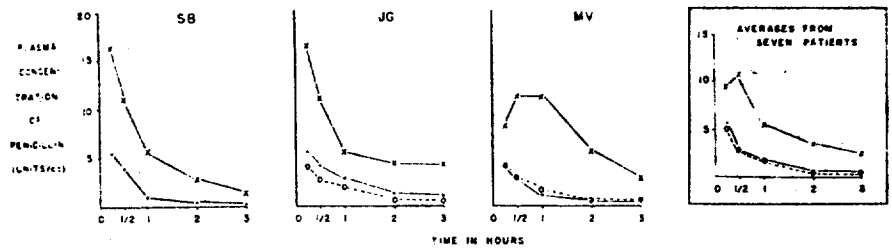
COMPARISON OF THE EFFECTS OF BENZOIC ACID AND CARONAMIDE UPON PENICILLIN DOSE RESPONSE CURVES



Effect of benzoic acid 2Gm/3hrs. compared with effect of caronamide 2Gm/3hrs. (4 patients)



Effect of benzoic acid 3Gm/3hrs. compared with effect of caronamide 3Gm/3hrs. (3 patients)

LEGEND	
Penicillin, 200,000 u. intramusc. at zero time	Each patient was his own control. Several days intervened between the administration of benzoic acid and caronamide. Patients JJ, PR, JC, and JA were given benzoic acid before caronamide, patients SB, JG, and MV received caronamide before benzoic acid.
—— penicillin alone	
o---o penicillin and benzoic acid	
x—x penicillin and caronamide	

FIG. 1.

was observed. It was suggested that the results reported by Bronfenbrenner and Favour are largely attributable to fluid and salt restriction. The desirability of lowering the daily urinary output to as little as 400 to 600 cc is open to question, especially if patients are suffering from febrile illness. If, however, there is no contraindication to fluid restriction, it can be anticipated that the enhancing effect of dehydration could be superimposed upon the effect of caronamide herein reported.

Spaulding, Bondi and Early[6] reported favorably upon the effect of sodium benzoate in elevating plasma concentrations following the oral administration of penicillin, unrestricted fluid and food intake, and a small dose of sodium benzoate. Bronfenbrenner and Favour indicated that, "on the normal diet, 2.5 g of benzoic acid equalled 6 g of sodium benzoate in raising serum penicillin level." Thus, on an unrestricted diet, Spaulding, Bondi and Early observed a positive effect of a small dose of sodium benzoate, whereas Bronfenbrenner and Favour using a larger dose of benzoic acid, which they regard as a more effective drug than sodium benzoate, observed only a slight effect.

The prolonged enhancing effect of sodium benzoate reported by Bohls, Cook and Potter[5] has been questioned by Spaulding, Bondi and Early on the basis of certain shortcomings of the penicillin assay methods used.

It is quite clear that the few data available on the effects of benzoic acid and sodium benzoate do not permit final evaluation of these two agents as adjuncts to penicillin therapy. If, as indicated by Bronfenbrenner and Favour, restriction of fluid is required to demonstrate an enhancing effect of benzoic acid, use of this agent would have certain disadvantages in the treatment of febrile diseases.

Hippuric acid is formed both in the liver and kidneys by the conjugation of benzoic acid and glycine. Although it is postulated that the hippuric acid thus formed acts in a manner similar to para-aminohippuric acid, it is extremely doubtful whether the amount of circulating hippuric acid following benzoic acid administration is sufficient to inhibit by "mass action" the tubular excretion of penicillin. It is conceivable that benzoic acid may be conjugated in the kidneys and give rise to a "local" high hippuric acid concentration, but this is not subject to proof at present.

In the light of the data here reported, it is our opinion that benzoic acid has no effect upon penicillin plasma concentrations when given in the amounts described.

Conclusions. Benzoic acid given in doses of 16 and 24 g per day to patients on unrestricted diets failed to increase penicillin plasma concentrations. Caronamide given to the same patients in the same doses showed a uniform and marked enhancing effect, averaging five times the control values.

The authors wish to express their indebtedness to Miss Elizabeth Hughes, Department of Bacteriology, Sharp & Dohme, Inc., Glenolden, Pa. who was responsible for all penicillin assays herein reported, and to Doctors Wm. H. Erb, Joseph C. Yaskin, A. Ornsteen, and Sherman F. Gilpin of the staff of the Philadelphia General Hospital, Philadelphia, Pa. for their courtesy in allowing us to use their patients for these studies.

Oral and Parenteral Use of Aluminum Penicillin Mixtures in the Treatment of Gonorrhea

S. W. Bohls, M. D.,[1] E. B. M. Cook, M. A.,[2] and R. T. Potter, M. D., M. P. H.[3]

The antibacterial action of penicillin on Neisseria gonorrhoeae was pointed out by Fleming (*1*) in his earlier publications on this antibiotic. Hobby, Meyer, and Chaffee (*2*) in 1942 placed the gonococcus high on their list of organisms susceptible to penicillin, but it was not until early in 1943 that Herrell, Cook, and Thompson (*3*), and Mahoney, Ferguson, Buchholtz, and Van Slyke (*4*) reported on the clinical use of penicillin in gonorrhea cases. Herrell and his coworkers used the intravenous drip method while Mahoney and his group used intramuscular injections. Early reports from the Committee on Chemotherapeutics and Other Agents of the National Research Council (*5*) further confirmed the good results in the treatment of gonorrhea with penicillin, and since that time the work of numerous groups on thousands of cases has established penicillin as a specific therapeutic agent for this disease.

The problem resolved itself into a matter only of adequate dosage and administration of the drug for gonorrhea and its complications. From even the earlier reports it was evident that adequate blood levels must be maintained for a requisite number of hours to obtain cures. The dosage and treatment interval had to be carefully adjusted to obtain this therapeutic interval. The most commonly used treatment schedules for gonorrhea at the present time are 150,000 to 200,000 units of sodium penicillin given intramuscularly in 3 injections at 2- to 3-hour intervals. The penicillin is dissolved in saline or distilled water and the injections are usually made in the gluteal or deltoid muscles. Such schemes of treatment result in a high percentage of cure but still require several injections.

Various methods for prolonging the therapeutic action of injections of penicillin have been reported. Among the more successful methods have been the use of penicillin-oil emulsions by Raiziss (*6*), penicillin beeswax-oil mixtures by Romansky and Rittman (*7*), and the use of penicillin-gelatin mixtures in conjunction with a vasoconstrictor by Parkins, Wiley, Chandy, and Zintel (*8*). In common with other investigators of this problem of prolonging blood levels of penicillin, we were desirous of finding a method which, by delaying absorption and consequent maintenance of blood levels, could be used so that the treatment of gonorrhea as well as other infections could be carried out in the clinic or in the office of the physician. Three lines of investigation have been followed with this objective in mind.

1. It was found that delayed absorption resulting in more prolonged blood level time occurred when penicillin was combined with buffered aluminum potassium sulfate before injection. Bohls and Cook (*9*) reported that a single intramuscular injection of 50,000 units of aluminum penicillin mixture would maintain a therapeutic level for 6 to 9 hours. Various dosages of the aluminum penicillin mixture in single and double doses were em-

The authors wish to acknowledge the assistance given by the following doctors who furnished advice and to the patients who helped to make the clinical study of the oral penicillin tablets possible: T. E. Dodd, M. D., M. P. H., Director, Division of Venereal Disease Control, State Health Department; R. J. Burkhart, M. D., Medical Officer in Charge, Riverview Hospital, San Antonio, Texas; W. S. Green, M. D., Chief, Division of Venereal Disease Control, San Antonio City Health Department; and M. M. Goldberg, M. D., Medical Officer in Charge, Riverside Hospital, Waco, Texas.

The authors also wish to acknowledge the cooperation of Hynson, Westcott, and Dunning, Inc., Baltimore, Md., who prepared and furnished the oral penicillin tablets for this study.

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[2] Senior Immunologist, Biologics Division, Bureau of Laboratories

[3] Passed Assistant Surgeon, U. S. Public Health Service, Field Consultant, Division of Venereal Disease Control.

ployed on cases of gonorrhea. The penicillin blood levels obtained in this series of patients are presented in table 1.

2. A second line of approach toward prolonging blood levels of penicillin was a combination of delayed absorption with the aluminum penicillin mixture and renal blockage by means of oral administration of benzoic acid as reported by Bohls and Cook (*10*). Tables 2 and 3 present the results of this study on a series of patients. Various agents have been reported in studies on selective renal blocking of penicillin excretion by the kidneys. Rammelkamp and Bradley (*11*) used injections of diodrast; Beyer, Flippen, Verwey, and Woodward (*12*) used para-amino-hippuric acid; and Bronfrenbrenner and Favour (*13*) used benzoic acid with intravenous and intramuscular injections of plain penicillin.

TABLE 1.—*Trial dosages of aluminum penicillin mixtures for intramuscular injection in the treatment of gonorrhea*

Patient	Dosage in units	Blood serum concentration of penicillin in units per milliliter				Result 24-48-hour culture
		3 hours	6 hours	9 hours	12 hours	
1	*300,000	1.0	0.5	0.03	0.03	Negative.
2	*160,000		.25	.125	0	Do.
3	*150,000	1.0	.125	.03	0	Do.
4	*100,000	.25	.125	.03	0	Do.
5	*75,000	.25	.125	0	0	Do.
6	*50,000	.25	.125	0	0	Do.
7	*25,000	.125	0	0	0	Do.
		Hours after first injection				
		9	12	15	18	35
8	**200,000	0.125	1.0	0.125	0.125	0.06	Negative.
9	**150,000	.125	.25	.125	.25	0	Do.
10	**100,000	.125	.25	.125	.03	0	Do.
11	**75,000	.125	.25	.125	.03	0	Do.
12	**50,000	.25	.125	.125	.125	0	Do.
13	**25,000	0	.125	.03	0	0	Do.

*Single injection.
**Given in 2 injections, 9 hours apart.

These tables show that the addition of benzoic acid caused a definite prolongation in the penicillin blood level maintained. The combined action of the 2 agencies resulted in holding blood levels through 24 to 26 hours with 2 injections of 25,000 units each at 7- to 8-hour intervals and assayable levels through 28 hours with 1 injection of 50,000 units.

3. The third type of investigation carried out was with an oral penicillin preparation. Penicillin has been administered orally by many investigators with conflicting results. It has been established that penicillin can be absorbed from the gastro-intestinal tract, but that it is at least partially destroyed by the hydrochloric acid of the stomach (*14*). Penicillin has been administered orally alone (*15*), with buffers (*16*), in various vehicles (*17*), in various protective coatings (*18*), and in chemical combinations (*19*). Therapeutic serum levels have been obtained and maintained by penicillin administered orally. To obtain therapeutic penicillin serum levels, however, it has been necessary to give larger oral doses than when the drug is given parenterally.

A number of methods have been investigated to prolong the period during which a therapeutic penicillin blood level can be maintained following a single oral dose. Investigations of methods for prolonging the penicillin blood levels have taken 2 directions: (1) diminishing the absorp-

Five experiments were performed on one normal subject (Nos. 2, 3, 12, 13, 13a). The data are presented in Figure 1. The ingestion of 10 gms. of benzoate resulted in a larger hippuric acid excretion and a greater absolute drop in plasma glycine than the administration of 6 gms. Furthermore, the ingestion of 10 gms. of glycine the night prior to the test resulted in a larger hippuric acid excretion, without, however, a greater decline in plasma glycine than obtained following the administration of 10 gms. of benzoate alone. The administration of glycine half an hour after the

TABLE 1

The effect of benzoate on plasma glycine and on hippuric acid formation

	No.	Sub.	Sex	Age	Diagnosis	Na benz. taken	Hipp. acid excretion		Urine vol.
						gms.	*gms.*	*hrs.*	*cc.*
No supplementary glycine	1	M. G.	F	21	Bronchial asthma	10			
	2	A. V.	M	35	Normal (1-9-47)	10	5.10	3	440
	3	A. V.	M	35	Normal (5-12-47)	6	3.77	3	360
	4	F. B.	F	44	Essential hypertension	10			
	5	W. T.	M	60	Arterioscler.; heart disease, congestive failure	6	3.48	3	355
	6	L. S.	M	70	Infectious hepatitis	6	4.15	3	950
	7	I. G.	M	43	Infectious hepatitis	6	4.28	3	310
	8	S. R.	M	49	Car. of pancreas	6	4.2	3	360
	9	E. A.	F	32	Nutrit. hypoproteinemia	6	2.03	4	[illegible]
	10	Th. W.	M	52	Virus pneumonia; malnutrition. Reconvalescent (5-6-47)	6	2.61	3	[illegible]
	11	P. J.	M	25	Rheumatoid arthritis	6	1.92	3	380
Additional glycine given*	12	A. V.	M	35	Normal (1-29-47); 10 gms. glycine taken night before test	10	7.69	3½	300
	13	A. V.	M	35	Normal (5-27-47); 3.12 gms. glycine taken ½ hour after ingestion of 6 gms. Na benzoate	6	5.18	3½	435
	13a	A. V.	M	35	Normal (6-2-47); 3.12 gms. glycine w/o Na benzoate				
	14	Th. W.	M	52	Virus pneumonia; malnutrit. Reconvalescent. 3.12 gms. glycine taken simultaneously w/o gms. Na benzoate	6	4.92	3	640

TABLE 1—*Continued*

	No.	Sub.	Plasma glycine—*mgm. per cent* Fasting					Plasma glycine—*mgm. per cent* After benzoate ingestion													Plasma glycine decrease	
			Time in minutes					Time in minutes														
			−60	−45	−30	−15	0	15	30	45	60	75	90	105	120	135	150	165	180	195	*mgm. per cent*	*per cent*
No supplementary glycine	1	M. G.		2.66			2.47			1.82				1.65							0.82	33
	2	A. V.	2.61				2.56		2.27				1.99				1.91				0.65	21
	3	A. V.					1.65				1.27				1.33				1.30		0.35	21
	4	F. B.					2.06			1.56				1.47				1.48			0.58	28
	5	W. T.					2.10				1.37				1.15				1.42		0.68	32
	6	L. S	2.19				2.17			1.76									1.91		0.26	12
	7	I. G.			1.91		1.99				1.61				1.61				1.44		0.55	[illegible]
	8	S. R.		1.99			1.85		1.75						1.45						0.40	22
	9	E. A.			3.86		3.90		2.31						2.47					1.86	2.04	52
	10	Th. W.		1.44			1.33				0.92				0.88				0.84		0.49	37
	11	P. J.			1.26		1.29				1.22				1.20				1.28		0	0
Additional glycine given*	12	A. V.		2.49			2.41				2.19				2.10				1.82		0.59	[illegible]
	13	A. V.					2.02		†		2.31				3.80				2.00			
	13a	A. V.					1.69†		5.81				2.78				1.91					
	14	Th. W.					1.73†				2.89				2.60				2.00			

* Glycine administered orally. † Time of glycine ingestion.

Ten patients with culturally positive gonorrhea were treated with a single dose of 100,000 units of penicillin in such oral tablets. The patients in this group were cultured 48 hours after they were given the medication and at that time 4 were positive and 6 negative. The blood levels of this group at ½, 1, 1½, 4, 8, and 24 hours averaged 0.141, 0.327, 0.375, 0.146, 0.049, and 0.023 units per milliliter of blood serum, respectively. All the penicillin blood serum assays mentioned in this paper were done by one of us (Cook) by the tube method of Randall, Price, and Welch (*21*) and the plate cup method of Schmidt and Moyer (*22*). The results of this study are shown in table 4.

A second group of 15 similar patients was treated with a single dose of 200,000 units of penicillin also given in oral tablets. Table 5 shows the data on this group. These patients, like those in the first group treated with oral tablets, were cultured 48 hours after the medication was given, and at that time only 1 positive gonorrhea culture was found. The blood levels of this group at ½, 1, 1½, 4, 8, and 24 hours averaged 0.391, 0.277, 0.576, 0.281, 0.178, and 0.078 units per milliliter of blood serum, respectively.

TABLE 4.—*Result of 100,000-unit oral penicillin therapy of gonorrhea*

Patient	Blood serum concentration of penicillin in units per milliliter (Hours after treatment)						Result 24 48-hour culture
	½	1	1 ½	4	8	24	
1	0.07	0.125	0.125	0.25	0.07	0.017	Negative.
2	.25	.5	.5	.25	.017	.035	Positive.
3	.07	.07	.25	.125	.125	0	Negative.
4	.07	.5	.5	.25	.017	0	Do.
5	.035	.25	.5	.07	.035	0	Do.
6	.125	.25	.25	0	.017	.035	Do.
7	.125	.5	.5	.25	0	0	Do.
8	.5	.5	.5	.125	.125	.07	Positive.
9	.035	.07	.125	.017	.017	.035	Do.
10	.125	.5	.5	.125	.07	.035	Do.
Average	.141	.327	.375	.146	.049	.023	

Additional test of cure cultures were not made on these first 2 series of orally treated patients because of the shortage of bed space in the 2 rapid treatment centers where the trials were made. The failures were treated by routine injection of sodium penicillin and all responded satisfactorily.

Even though the results with the oral tablets were merely suggestive since we were unable to obtain sufficient follow-up cultures, they were encouraging. Accordingly, it was decided to give a further clinical trial with the oral tablets. For this purpose, a series of 49 patients was given 100,000 units of penicillin each in this form. All the patients in this group were women treated in the Quarantine Clinic of the San Antonio City Health Department. This clinic afforded an ideal location in which to evaluate the therapeutic effectiveness of the medication, for in it there was no possibility of reinfection during the period of investigation. The patients represented promiscuous women apprehended by the police on morals charges and consequently there was included in this group more than the average number of cases of chronic gonorrhea with complications. All cases admitted to the study had positive cultures for the gonococcus before treatment was instituted.

After having been diagnosed, 13 of the patients were given 100,000 units of penicillin in the tablet form at 7 to 8 a. m. and 3 to 4 p. m. on 2 successive days. Follow-up cultures as tests of cure were then taken at 48, 72, 96, and 120 hours later. Among this group there were 3 failures, giving a failure rate of 23.1 percent. Penicillin blood levels were not obtained on any of the 49 patients treated in the Quarantine Clinic.

TABLE 5.—*Result of 200,000-unit oral penicillin therapy of gonorrhea*

Patient	Blood serum concentration of penicillin in units per milliliter (Hours after treatment)						Result 48-hour culture
	½	1	1½	4	8	24	
1	0.25	0.5	0.25	0.5	0.07	0.5	Negative.
2	1.0	.125	.5	.25	.07	.5	Do.
3	1.0	.07	.5	.25	.017	.0	Do.
4	1.0	.017	.5	.07	.017	.035	Do.
5	.035	.125	1.0	.5	.017	.0	Do.
6	.5	.07	.07	.5	1.0	.0	Do.
7	1.0	.125	1.0	.25	1.0	.017	Do.
8	.07	.5	1.0	.5	.07	.07	Do.
9	.017	.5	.125	.5	.125	.017	Do.
10	.125	.125	1.0	1.0	.0	.035	Do.
11	.25	.5		.5	.125	.0	Do.
12	.125	.25	.5	.125	.07	.0	Do.
13	.125	.25	.125	.5	.035	.0	Do.
14	.25	.5	.5	.017	.025	.0	Do.
15	.125	.5	1.0	.25	.035	.0	Positive.
Average	.391	.277	.576	.381	.178	.078	

It was felt by the authors, on the basis of the work previously mentioned, that although penicillin blood levels were probably maintained over a relatively long period of time with the treatment scheme employed, the levels achieved were not sufficiently high to "cure" in all cases. The second portion of this group of patients was therefore given a similar total dosage of 100,000 units, but it received 200,000 units at approximately 7 to 8 a. m. and at 3 to 4 p. m. Thus they received 200,000 units initially followed by an additional 200,000 units 7 to 8 hours later. Thirty-six patients received this treatment and each had cultures taken as tests of cure at 48, 72, 96, and 120 hours. There was only 1 failure among the 36, giving a failure rate of only 2.8 percent.

No toxic reactions were experienced among the total 74 patients treated by the oral penicillin tablets in the 4 different schemes employed. The tablets each contained 25,000 units of penicillin. The preparation is quite stable and may be kept at room temperature, although storage under refrigeration is advised. We gave the tablets without relationship to meals or fluid intake. No effort was made to influence the physical activity of the patients while they were being treated.

This is a preliminary report and the 4 different oral schemes of therapy used were chosen arbitrarily and undoubtedly do not represent either the optimum amount of penicillin or the best time-dosage schedule. Further trial and experience are indicated before this desideratum can be reached. A more complete account of this oral aluminum penicillin benzoic acid research will be published in the article by Bohls, Cook, and Dodd, given as reference (26). Three cases of primary and 1 of secondary syphilis were given oral penicillin tablets in experimental dosages and all showed rapid and definite clinical improvement.

SUMMARY

1. The use of an aluminum penicillin preparation by injection with and without the addition of oral benzoic acid is discussed in the treatment of gonorrhea. In this preparation, the aluminum causes delayed absorption and the benzoic acid inhibits renal excretion of the penicillin.

2. The use of an oral aluminum penicillin tablet containing sodium benzoate in the treatment of gonorrhea is discussed. A dose of 200,000 units of this preparation maintained a penicillin blood level for over 24 hours.

3. Two hundred thousand units of this oral penicillin given at 8 a. m. and repeated at 4 p. m. for a total dose of 100,000 units resulted in a "cure rate" of 97.2 percent in a series of female gonorrhea patients.

4. No untoward signs or symptoms were experienced with this type of penicillin therapy.

5. This is a preliminary report and the optimum time-dosage schedule of this type of oral penicillin therapy in the treatment of gonorrhea has not as yet been ascertained.

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BENZOIC ACID AND BENZOATES ELIMINATION IN METABOLISM

Paper by Mssrs. BORDAS, FRANÇOIS-DANVILLE & ROUSSEL presented by Mr. A. d'Arsonval

Benzoic acid and certain of its salts have been recommended for a number of years for the preservation of food. This preservative possesses real antiseptic properties; papers published by BUCHOLTZ, OKOLOW, KLEBS MIQUEL are unanimous in recognizing its microbicide value.

Sodium benzoate has hardly any taste, is odorless, can be introduced into food in sufficiently high doses without modifying noticeably the the organoleptic characteristics.

We shall recall that sodium benzoate is very much in use in the treatment of acute and chronic throat and respiratory diseases; it is also recommended as antiseptic of the genito-urinary system and as cholagogue in gall stones.

It is therefore a very precious remedy, which, by the way, appears in the pharmacopoeia.

The physiological effects of alkali benzoates have been thoroughly studied and if certain authors like WEISKE, WOGL, Von JAKSCH do not grant them toxic effects, others, on the contrary, consider that the ingestion of sodium benzoate for instance provokes nausea and vomiting in certain people (MEISSNER & SHEPARD) and in higher doses provokes a total stoppage of digestive processes (OKOLOW).

WILEY is of the opinion to prohibit its use for food preserving.

The works of RUSSEL H. CHITTENDEN, deJOHN H. LONG and CHRISTIAN A HERTER relating to the effects on the human organism produced by the intake of small and massive doses of the benzoate of sodium do not appear adverse to the use of alkali benzoates as preservatives. We have to add that these conclusions are not admitted by all hygienists. The experiments of the American authors were criticized because they gave variable doses of the benzoate to young students from 20 to 27 (one only was 45 years old).

The studies by BROUARDEL, GERMAIN SEE on salicylic acid and Na salicylate have shown that elimination by the kidneys is by no means equally rapid and complete in all subjects and that there can be accumulation in the organism varying with age and certain morbid states (Dr.MARY DURAND).

It was important to first verify if the Na benzoate whose elimination process bears, as is known, certain analogies with Na salicylate (BERTAGNINI) would not cause similar disorders in the general elimination mechanism by means of the kidneys.

We had older people ingest in a single dosis 2 gr of Na benzoate : 10 from 60 to 68 years of age; 4 from 52 to 58; 1 of 49; 1 of 35 and 2 of less than 25. Urines were collected and analyzed. Three hours after absorption of the substance hippuric acid and benzoic acid were found in all subjects.

Elimination took place during 1 day after ingestion in 3 subjects;

2 days after ingestion in 13 subjects and 3 days after ingestion in 2 subjects.

As is seen elimination varies in different individuals. Of the 3 subjects who eliminated in one day, one was 25 years old. Generally, elimination occurred 2 days after ingestion.

We made a second series of experiments giving 2 gr. of Na benzoate for 8 consecutive days, e.g. 16 gr. to 7 subjects varying in age.

Elimination took place as follows:

a) 1 day after complete drug regime benzoic acid elimination: 0;

b) during 2 days after complete regime elimination: 1;

c) during 3 days after complete regime elimination: 6

The accumulation is even more marked here since Na benzoate was not completely eliminated until after 3 days and this by 6 out of 7 subjects.

The ingestion of Na benzoate in a continuous fashion as would be the case if its use for the preservation of food were generalized would therefore be susceptible to provoke more or less serious disturbances because of its accumulation in the organism.

Patients under benzoate of sodium treatment are examined by their doctor. The action of the medication is followed and observed daily as noted by DUBRISAY; things are different in the case of healthy or so-called healthy people who, in the belief of taking nourishment, are made to absorb salicylic or benzoic antiseptics, that is real drugs.

ACTA CHEMICA SCANDINAVICA 11 (1957) 541—544

The Effect of Salicylic Acid, Benzoic Acid and some of their Derivatives on Oxidative Phosphorylation

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Salicylic acid (10^{-2} M) and benzoic acid (10^{-1} M) cause an uncoupling of oxidative phosphorylation from respiration in isolated rat liver mitochondria. Similar results are obtained with 5-nitro-salicylic acid and with methyl benzoate.

The growth inhibiting effect of salicylic and benzoic acids on microorganisms has been the subject of many studies with the purpose of elucidating the mode of action of these compounds. In recent years investigations have been published which call attention to the possibility that the growth inhibiting effect might originate, at least to some extent, in an inhibition of the formation or utilization of high energy compounds [1-5]. In order to obtain further evidence, the effect of salicylic and benzoic acids and some of their derivatives on the oxidative phosphorylation in isolated rat liver mitochondria was studied in this work.

EXPERIMENTAL

Preparation of mitochondria. The preparation of mitochondria was carried out according to the method of Schneider and Hogeboom [6]. The rats were killed by decapitation and the livers put in 0.25 M ice-cold sucrose as fast as possible. In this solution the liver was cut into small pieces and rinsed several times with sucrose solution. The homogenization was carried out in a Potter-Elvehjem homogenizer. The homogenate was diluted to *ca.* 100 ml with 0.25 M sucrose and centrifuged at 1 600 g for 10 minutes. The supernatant was decanted and recentrifuged at 4 100 g for 20 min. The mitochondria were washed twice with *ca.* 100 ml sucrose each time.

Materials. Yeast hexokinase was used as a stock solution which had been prepared according to Berger *et al.*[7] with minor modifications [8]. ATP, as the crystalline disodium trihydrate, had been furnished by the Sigma Chemical Company, St. Louis, Mo., U.S.A., and $H_3{}^{32}PO_4$ by the Radiochemical Centre, Amersham, England. All solutions were prepared in glass-distilled deionized water.

Incubation procedure. Mitochondria from one liver were suspended in 13.3 ml 0.25 M sucrose containing 116 mg $K_2H^{32}PO_4$, 144 mg glucose and 12 mg ATP. The pH was adjusted to 7.5 by means of glass electrode. To this suspension 1.3 ml 0.1 M $MgCl_2$ was added and the suspension was diluted to 20 ml with 0.25 M sucrose. In all experiments 1.5 ml of this suspension was added to each vessel.

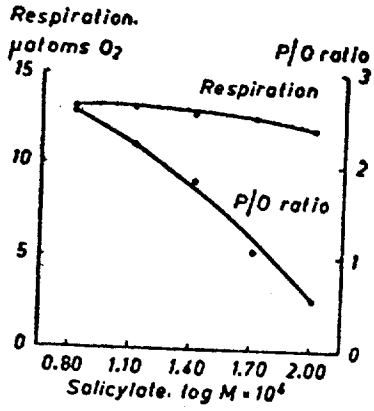

Fig. 1. The effect of increasing concentrations of salicylate on oxidative phosphorylation. Each vessel contained besides the suspension described in the experimental part 30 µmoles potassium α-ketoglutarate as substrate and potassium salicylate in the concentrations indicated in the figure. Experimental conditions: gas phase, air; temp., 30°C; time of incubation, 20 min.

Analytical procedures. Oxygen consumption was measured by the conventional Warburg technique. The described suspension and neutralized solutions of the different inhibitors were added to the main compartments of the Warburg vessels, substrates and hexokinase (0.1 ml of the stock solution) to the sidearms. The centre wells contained 0.2 ml 2 M KOH, absorbed on a roll of filter paper. The final volume of solution in the vessels was adjusted to 2 ml with 1.15 % KCl. Manometer readings were made at 5 min invervals, starting 5 min after the addition of substrate and hexokinase from the sidearms. Oxygen consumption for the period 0—5 min was calculated by extrapolation. At the end of the experiments, enzymatic activity was terminated by the addition of 0.2 ml 5 M H_2SO_4. Phosphate uptake was determined as the ratio of organic phosphate formed to inorganic phosphate added by the isotope distribution method described by Lindberg and Ernster [9].

RESULTS

The effect of increasing concentrations of salicylate on oxidative phosphorylation and oxygen consumption with α-ketoglutarate as substrate is illustrated by Fig. 1.

The figure shows that salicylate at a concentration of about 1×10^{-2} M almost completely inhibits phosphorylation, while the inhibition of oxygen consumption is negligible. Similar results are obtained if α-ketoglutarate is replaced by pyruvate + malate, succinate, or glutamate.

Table 1. The effect of salicylate on oxidative phosphorylation.

Additions	Without hexokinase	With hexokinase	
	Oxygen µatoms	Oxygen µatoms	P/O ratio
None	4.6	11.2	2.5
DPN + cytochrome c	5.3	11.7	2.3
Salicylate	9.4	8.5	0.6
Salicylate + DPN + cytochrome c	10.3	9.6	0.5

The final concentrations of the substances added to the suspension of the mitochondria were as follows: DPN, 6.8×10^{-4} M; cytochrome c, 10^{-5} M; potassium salicylate, 10^{-2} M. Each vessel contained 30 µmoles potassium α-ketoglutarate as substrate. Experimental conditions as in Fig. 1.

Table 1 exemplifies an experiment which shows that salicylate, at the concentration mentioned above, is able to induce an almost maximum level of respiration in the absence of hexokinase. This is a further indication that salicylate uncouples phosphorylation from respiration, since, in this case, respiration proceeds in the absence of a terminal phosphate acceptor.

Experiments with benzoate showed that this substance had a similar effect, which, however, became noticeable only at still higher concentrations than those which were necessary in the case of salicylate. The results of a typical experiment with benzoate are shown in Table 2. The relatively high concentrations of benzoate caused a partial inhibition of respiration along with the effect on phosphorylation. It was possible to bring up the respiration to the original level by the addition of DPN and cytochrome c, without, however, a simultaneous recovery of the phosphorylation. This indicates that the inhibition of respiration is a secondary effect, due to a release of DPN and cytochrome c from the mitochondria.

Similar pictures are obtained if glutamate is replaced by other substrates such as succinate and α-ketoglutarate.

Table 2. The effect of benzoate on oxidative phosphorylation.

Additions	Without hexokinase	With hexokinase	
	Oxygen μatoms	Oxygen μatoms	P/O ratio
None	3.5	12.3	2.1
DPN + cytochrome c	6.7	12.2	2.0
Benzoate	8.2	10.7	1.0
Benzoate + DPN + cytochrome c	8.7	12.1	0.9

Experimental conditions as in Fig. 1. Each vessel contained 30 μmoles potassium glutamate. DPN and cytochrome c were added at the same concentrations as in Table 1. The benzoate concentration was 8×10^{-2} M.

In order to further elucidate the mode of action of salicylate and benzoate on oxidative phosphorylation, the effect of some related substances was investigated. Of these compounds 5-nitro-salicylic acid (which differs from 2,4-dinitrophenol only by the replacement of one of the nitro groups by a

Table 3. The effect of 5-nitro-salicylic acid on oxidative phosphorylation.

Substrate	Without 5-nitro-salicylic acid		With 10^{-3} M 5-nitro-salicylic acid		With 10^{-2} M 5-nitro-salicylic acid	
	Oxygen μatoms	P/O ratio	Oxygen μatoms	P/O ratio	Oxygen μatoms	P/O ratio
Glutamate	13.4	2.3	12.8	1.8	11.1	0.14
Succinate	13.8	1.5	13.9	1.2	4.8	0.15

Each vessel contained 30 μmoles substrate. Experimental conditions as in Fig. 1.

carboxyl group) and methyl benzoate inhibited phosphorylation to about the same extent as did salicylic acid, while 4-hydroxy-salicylic, 5-hydroxy-salicylic and 4-amino-salicylic acids (PAS) were without effect. Table 3 shows the results of an experiment with 5-nitro-salicylic acid.

DISCUSSION

It is evident from the results described in this paper that salicylic and benzoic acids produce an effect on oxidative phosphorylation, which is reminiscent of that obtained with 2,4-dinitrophenol. The concentrations required to obtain a marked effect are of the same magnitude as those required for a complete suppression of bacterial growth at the pH in question (7.5). As is well known, however, the growth inhibiting effect of salicylic and benzoic acids increases as the pH is lowered. This is probably due to the fact that the undissociated molecules of these acids are more lipophilic than the respective anions and, thus, more easily taken up by the bacteria. Such a correlation is well conceivable to prevail even in the present case, where an increasing lipid solubility might facilitate the penetration of these inhibitors into the mitochondrial structure. This point is, however, hard to test in experiments with isolated mitochondria, which lack the mechanism by which bacterial cells can make themselves independent at least to some extent of the pH of the environment. A lowering of the pH in such experiments may thus render oxidative phosphorylation impossible.

This work is part of an investigation supported by *Statens Naturvetenskapliga Forskningsråd* and *Statens Tekniska Forskningsråd*.

I wish to express my sincere gratitude to Professor Olov Lindberg for allowing me to perform these experiments at his Institute and to Dr. Lars Ernster for indispensable advice and valuable discussions throughout the work. I also wish to thank Professor Gösta Ehrensvärd for providing me with samples of some of the inhibitors and for stimulating discussions.

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Received January 12, 1957.

SOME ASPECTS OF INTERMEDIARY PROTEIN METABOLISM

THE exogenous origin of creatine was first demonstrated by Levene and Kristeller in 1909 in their protein feeding experiments with cases of muscular dystrophy. In 1929, we reported[1] that glycine fed to patients with muscular dystrophy produced a marked rise in creatine excretion (confirmed by Thomas *et al.*,[2] and others), whereas glutamic acid and cystine had no effect. Studies with a number of other amino acids and various other substances were also reported. The effect of the removal of glycine and of glutamic acid from the metabolic mixture through the feeding of benzoic acid and phenylacetic acid, respectively, was investigated. It was found that the former produced a marked drop in creatine, while the latter was without effect. We are inclined to infer from our experiments that the feeding of brombenzene, which removes cysteine from the metabolic mixture, would be without effect on creatine excretion. Owing to the possible toxicity of the substance we intend to carry out suitable animal experiments instead. The chart shows only the essential results of a few representative experiments.[3]

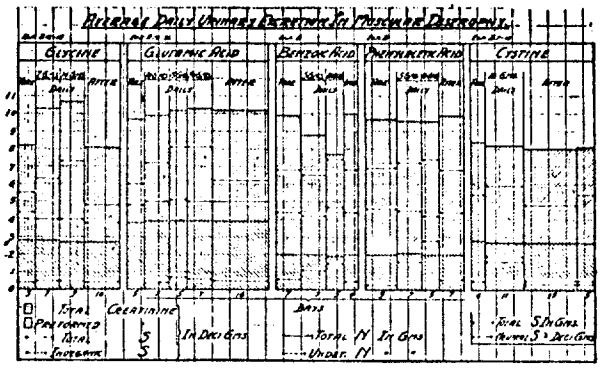
Hippuric acid, phenylacetyl glutamine and bromphenyl mercapturic acid formation: The finding that the feeding of benzoic acid and phenyl acetic acid in proper amounts may have no effect upon the total nitrogen catabolism, although there are appreciable amounts of glycine and glutamic acid, respectively, lost to the body as a result of "Abfang" processes,

[2] O. Warburg and W. Christian, *Biochem. Zeits.*, 258: 496, 1933.

[1] *Am. Jour. Physiol.* 90, 296.

[2] *Z. physiol. Chem.* 214, 121.

[3] The significance of our detailed nitrogen and sulfur studies can not be discussed here, due to lack of space.



we believe, would indicate that these amino acids are originally present in the metabolic mixture in appreciable amounts and not especially synthesized for detoxification. This view is substantiated by our findings that creatine is markedly reduced by the benzoic acid and uninfluenced by the phenyl acetic acid and also by our observations regarding the respective influence of glycine, glutamic acid and cystine upon the excretion of creatine.

The rôle of glutathione in intermediary protein metabolism: It is a surprising fact that the only three amino acids—glycine, glutamic acid and cysteine—which seem to be available for processes of detoxification in the human are the same as those which form glutathione.[4] If glutathione supplies the amino acids for these processes of detoxification, then the unaltered level of protein catabolism accompanying such processes would seem to indicate that an appreciable part of normal protein catabolism goes *via* glutathione. It is possible that this view may be further supported by the finding that methionine replaces cystine in cystine deficient diets (Jackson and Block) and by the observation regarding the influence of methionine on the detoxification of brombenzene in such diets (White and Lewis).

One may conceive that the substances, which are being detoxified, first combine with the glutathione, thereby rendering it more unstable, as a result of which the detoxified compound splits off in combination with part of the original glutathione molecule. This change in stability would be in keeping with our findings regarding factors which influence the lability of the glutathione and cystine molecules.[5]

E.g., phenylacetic acid would combine with the α-amino group of the glutamic acid radical of glutathione, following which the side chain splits off completely at the α-carbon of the cysteine liberating phenylacetylglutamine.

[4] This view has been expressed previously by others (Waelsch, H., *Arch. Exp. Path.* 156, 356; Power, F. W., Washington Meeting, Am. Chem. Soc. (1933)).

[5] *Jour. Biol. Chem.*, 70, 381.

Shiple and Sherwin have called attention to the obviously close relationship between phenylacetylglutamine excreted by man and phenylacetylornithine excreted by birds. Ornithine in this latter compound may be conceived as derived from glutathione by a process in which the distant carbonyl group of the glutamic acid radical is reduced to form a nor-valine radical, which if split off together with the α-amino group of the cysteine would yield ornithine. Such mechanisms may have some bearing on uric acid and pyrrole formation. The possibility of glutathione being a source of ornithine is of interest in connection with the work of Krebs on urea formation and the reported activation of arginase by glutathione. The glutamic acid linkage in glutathione, which linkage is not easily split by enzymes, has long been considered as unusual. The reactivity of the distant carbonyl group of the glutamic acid radical in glutathione hardly has been investigated.

Bromphenyl mercapturic acid referred to above contains an acetyl group, which may be a remnant of the glutamic acid radical of glutathione, rather than the result of acetylation.

Creatine formation: Our experiments indicate that the guanidine group of creatine is synthetic in origin and that glycine is involved in creatine formation. Since glutathione is now known to contain glycine, its possible relation to creatine formation is obvious.

Creatinine formation: Creatinester hydrochloride and related compounds spontaneously form their respective anhydrides upon neutralization, the free esters apparently being quite unstable (unpublished experiments by Failey and Brand). This behavior of creatinester might suggest that in the animal organism creatinine is formed from a creatine compound in which the carboxyl group of creatine is not free but linked up as an ester, thioxy ester or amide.

Our finding in cystinuria[6] regarding an unusual creatinine metabolism accompanying this disturbance in sulfur metabolism is of interest in this connection. Studies regarding the therapeutic and metabolic effects of glutathione and of mixtures of its constituent amino acids in myopathies are being planned.

We shall consider more fully some of the conceptions of protein metabolism outlined above and their implications in a more detailed publication of our experiments.

This work was aided in part by a grant from the Chemical Foundation.

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[6] *Jour. Biol. Chem.*, 86, 315.

Biochemical Pharmacology, 1966, Vol. 15, pp. 994–995. Pergamon Press Ltd., Printed in Great Britain.

Glycine acyltransferase activity in developing rat liver

(*Received* 3 *March* 1966; *accepted* 31 *March* 1966)

THE detoxication of benzoic acid and similar substances in mammalian tissues is brought about in three stages.[1] Benzoyl adenylate is formed by pyrophosphate exchange with ATP. The adenylate moiety is then exchanged for coenzyme A to produce benzoyl-CoA. Finally, the latter reacts with glycine to give hippuric acid with the regeneration of free CoA. The first two reactions are catalyzed by a thiokinase, the last by glycine acyltransferase (E.C. 2.3.1.13[2]), the specificity of which is indicated by its systematic name: acyl-CoA: glycine N-acyltransferase. The three activities are found only in the mitochondrial fraction of liver and kidney.[3]

Hippuric acid synthesis by liver homogenates has been previously shown to vary with the age of the animal.[4] The present study demonstrates that the activity of the glycine acyltransferase component of the system also varies with the age of the animal and in a similar pattern.

METHODS

Rats of the Sprague-Dawley strain were fed a standard laboratory diet (D & G, The Price-Wilbolte Co.). The approximate time of conception was determined by vaginal smear the morning after an overnight period of mating. Fetal rats were obtained by hysterotomy under ether anesthesia. Older animals were killed by decapitation without anesthesia.

All animals 20 days of age or older were virgin females. In assays involving fetal and newborn animals the livers of one to three litters were used for each experiment. Freshly excised livers were

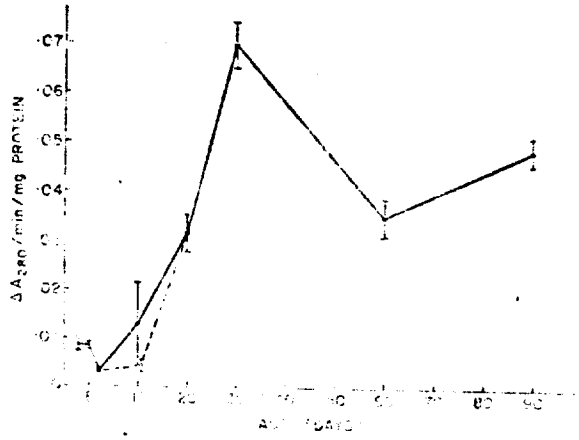

Fig. 1. The variation of glycine acyltransferase activity of rat liver with age. "B" indicates time of birth. [illegible] The broken line indicates values obtained when one markedly deviant result was omitted from the data in 10-day-old animals.

homogenized in 9 vol. of cold 0·25 M sucrose and the mitochondrial [illegible] fugation, washed four times, and made into acetone powder. [illegible] were assayed for glycine acyltransferase as described by Schachter and Taggart, [illegible] measuring the decrease in absorbance at 280 mμ as the thioester bond of benzoyl-CoA is cleaved. Protein was determined by the biuret reaction.

RESULTS

The enzyme activities of material prepared from animals of different ages are shown in Fig. 1. All points represent the results of six experiments except that five experiments were performed on 30-day-old animals, and four experiments were performed on 19-day fetuses and on 90-day-old animals. There is relatively little activity in fetal and early newborn life, and by the time of weaning (20 days) an appreciable increase has taken place. This proceeds to a maximum at 30 days of age then decreases to a small extent. This development pattern is similar to that described for hippuric acid synthesis by liver homogenates,[4] suggesting that glycine acyltransferase may be the rate-limiting step in the overall reaction. These experiments further define the probable biochemical basis for the reduced ability of newborn infants to metabolize *p*-aminobenzoic acid.[5]

Acknowledgements—The competent technical assistance of Margaret P. Holbrook is gratefully acknowledged. Supported by a grant from the United States Public Health Service (GM-06992).

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Biochem. J. (1970) **118**, 47–51
Printed in Great Britain

The Fate of Benzoic Acid in Various Species

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(*Received* 22 *January* 1970)

1. The urinary excretion of orally administered [^{14}C]benzoic acid in man and 20 other species of animal was examined. 2. At a dose of 50mg/kg, benzoic acid was excreted by the rodents (rat, mouse, guinea pig, golden hamster, steppe lemming and gerbil), the rabbit, the cat and the capuchin monkey almost entirely as hippuric acid (95–100% of 24h excretion). 3. In man at a dose of 1mg/kg and the rhesus monkey at 20mg/kg benzoic acid was excreted entirely as hippuric acid. 4. At 50mg/kg benzoic acid was excreted as hippuric acid to the extent of about 80% of the 24h excretion in the squirrel monkey, pig, dog, ferret, hedgehog and pigeon, the other 20% being found as benzoyl glucuronide and benzoic acid, the latter possibly arising by decomposition of the former. 5. On increasing the dose of benzoic acid to 200mg/kg in the ferret, the proportion of benzoyl glucuronide excreted increased and that of hippuric acid decreased. This did not occur in the rabbit, which excreted 200mg/kg almost entirely as hippuric acid. It appears that the hedgehog and ferret are like the dog in respect to their metabolism of benzoic acid. 6. The Indian fruit bat produced only traces of hippuric acid and possibly has a defect in the glycine conjugation of benzoic acid. The main metabolite in this animal (dose 50mg/kg) was benzoyl glucuronide. 7. The chicken, side-necked turtle and gecko converted benzoic acid mainly into ornithuric acid, but all three species also excreted smaller amounts of hippuric acid.

In a previous paper (Adamson, Bridges, Evans & Williams, 1970) it was shown that in man and certain Old World monkeys orally administered quinic acid was extensively aromatized apparently by gut bacteria to benzoic acid and excreted as hippuric acid. In certain other primates and lower animals this did not occur to any large extent. However, it was possible that in the lower animals the conversion of quinic acid into benzoic acid did occur, but that the latter was destroyed before it had the opportunity of being conjugated and excreted. The fate of [^{14}C]benzoic acid was therefore examined in man and 20 other species of animals, including three species of subhuman primates. In the present paper it is shown that all the species examined excrete orally-administered benzoic acid mainly as its conjugates and this suggests that benzoic acid is not destroyed in these animals. Some new information on the fate of benzoic acid in certain species has been found.

MATERIALS AND METHODS

Materials. [*ring*-^{14}C]Benzoic acid (395μCi/mg) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Benzoic acid, m.p. 122°C, and hippuric acid, m.p. 187°C, were purchased and purified. [*carbonyl*-^{14}C]Hippuric acid (0.022μCi/mg), L(+)-ornithuric acid, m.p. 182°C and $[\alpha]_D^{23}$ +11.9° (*c* 1 in 0.1M-NaOH), and benzoyl glucuronide, m.p. 180°C and $[\alpha]_D^{20}$ −25.6° (*c* 0.7 in water), were samples previously prepared in this laboratory. The purity of these compounds was checked by paper chromatography and, where possible, by radiochromatogram scanning.

Animals and dosing. The animals used are listed in Table 2. They were obtained from animal dealers mainly in the London area and were maintained on an appropriate diet. Sodium [^{14}C]benzoate was administered to the subhuman primates, the dogs and pigs mixed in food and to cats and ferrets dissolved in milk. The rodents, reptiles, fruit bats and hedgehogs received the compound dissolved in water by syringe into the stomach and the birds and rabbits by stomach tube. The animals were kept in metabolism cages appropriate to their size and their urine was collected daily in receptacles containing a few millilitres of saturated aq. $HgCl_2$ solution to prevent bacterial breakdown of conjugates. Birds were starved for 24h before the administration of benzoate to decrease the amount of faeces, and their excreta were collected on galvanized metal trays placed below their cages. All urines were kept frozen after collection until needed, but were analysed for ^{14}C as rapidly as possible after collection.

Chromatography. The R_F values and methods of detection of benzoic acid and its metabolites are given in Table 1.

Radiochemical techniques. The radioactivities of all

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Table 1. *R_F values and colour reactions of benzoic acid and its metabolites*

Whatman no. 1 paper with the descending method was used. The solvent systems were: *A*, butan-1-ol–ethanol–water–acetic acid (3:1:1:0.1, by vol.); *B*, butan-1-ol saturated with a solution of equal volumes of 1.5M-NH_3 and 1.5M-$(NH_4)_2CO_3$; *C*, butan-1-ol–acetic acid–water (4:1:2, by vol.). NR, naphtharesorcinol spray (Bridges, Kibby & Williams, 1965); DMAB, spray of *p*-dimethylaminobenzaldehyde in acetic anhydride (4%, w/v) containing a little sodium acetate, followed by gentle heating of the paper with a hot-air blower; DMAC, spray of *p*-dimethylaminocinnamaldehyde in acetic anhydride (0.05%, w/v), then heating the paper for 2min at 120°C. The dried paper was illuminated with u.v. light (254nm) from a Hanovia Chromatolite lamp: q, quenching of background fluorescence of the paper.

	R_F value			Colour reactions			Appearance in u.v. light
	Solvent *A*	Solvent *B*	Solvent *C*	NR	DMAB	DMAC	
Benzoic acid	0.92	0.45	0.90	—	—	—	q
Hippuric acid	0.83	0.35	0.83	—	Yellow	Red	q
Benzoyl glucuronide	0.62	0.23	0.67	Blue	—	—	q
Ornithuric acid	0.91	0.70	0.89	—	—	White on yellow background*	q

* Blue fluorescence in u.v. light.

urine samples (0.5–1.0ml) were counted in quadruplicate in a dioxan scintillator fluid (20ml) (Bridges, Davies & Williams, 1967). Faeces and tissues (1g) were homogenized in 10 parts (v/w) of water and then made up to 100ml in water. Radioactivities of 0.5ml samples of this solution were counted in triplicate in a gel dioxan scintillator system (Bridges *et al.* 1967) in a Packard Tri-Carb model 3214 scintillation counter. All samples were counted at 0°C and then recounted about a week later to allow for possible errors due to chemiluminescence. Radiochromatogram scans of the urine of every animal given [^{14}C]benzoic acid were prepared with a Packard model 7200 radiochromatogram scanner. The urine (0.1ml) was applied as a band to a strip (3.8cm wide) of Whatman no. 1 paper and dried. The chromatograms were developed during 15h in solvents *A*, *B* or *C* (see Table 1), and then scanned for ^{14}C.

When necessary benzoic acid and hippuric acid were determined by reverse isotope dilution, the acids being isolated from acidified urine and their radioactivities counted before and after conversion into their *p*-nitrobenzyl esters (Adamson *et al.* 1970).

RESULTS AND DISCUSSION

The results of our experiments on the fate of orally-administered [^{14}C]benzoic acid in man and 20 other species are summarized in Table 2. Most of the animals were given the compound at a dose of 50mg/kg, and in most cases 50–100% of the ^{14}C administered was excreted in the urine within 24h of dosing. All the ^{14}C excreted was present as compounds containing benzoic acid. The only animals in which the excretion of ^{14}C was relatively slow were the turtle, which excreted 12% and 39% in 2 and 3 days respectively, and the geckos, which excreted 32% and 39% in 2 and 3 days respectively.

The metabolites of benzoic acid in most species are hippuric acid and benzoyl glucuronide, although some species may produce ornithuric acid or benzoylarginine (see Williams, 1967*a*; Smith, 1968). Table 2 shows that in most of the herbivorous and omnivorous animals benzoic acid when given at 50mg/kg is excreted almost entirely (90–100%) as hippuric acid. These animals include man, rhesus monkey, squirrel monkey, capuchin monkey, pig, rabbit and the six species of rodents (rat, mouse, guinea pig, hamster, lemming and gerbil). The two pigs and two squirrel monkeys examined excreted some 10–20% of the ^{14}C eliminated in 24h as free benzoic acid, and it is possible that this could have arisen by decomposition of the relatively labile benzoyl glucuronide. The result for man is not strictly comparable with the other animals in this group, since the dose given was only 1mg/kg. However, the dose (70mg) was excreted by both subjects entirely as hippuric acid, and 97% of the ^{14}C administered was excreted within 4h after dosing and virtually 100% in 12h.

Three carnivorous species were examined, namely the cat, dog and ferret. In the cat the whole of the ^{14}C excreted was present as hippuric acid; this was not unexpected, since the cat has a defective glucuronic acid conjugation (see Williams, 1967*a*,*b*). Both the dog and ferret, however, excreted appreciable amounts of benzoyl glucuronide. It is known that in the dog the rate of mobilization of glycine for hippuric acid synthesis is relatively low compared with other species, and further, glycine conjugation occurs only in the kidney, whereas in the rat and rabbit for example it occurs in both the liver and the kidney. Therefore in the dog the tendency for benzoic acid to conjugate with glucuronic acid occurs at lower doses than in other

Table 2. *Metabolites of* [^{14}C]*benzoic acid in urine in various species*

Sodium [^{14}C]benzoate was administered orally as described in the text. The first 24 h urine (unless otherwise stated) of each animal was chromatographed on Whatman no. 1 paper (see Table 1 for solvents) and the paper scanned in a Packard radiochromatogram scanner. Where three or more animals were used the results are expressed as averages with ranges in parentheses. Where only one or two animals of a species were used the individual results are given. The amounts of the metabolites were estimated from the chromatogram scans as described in the text. M, male; F, female.

Species (no. and sex)	Family or other description	Dose: Benzoic acid (mg/kg)	Dose: ^{14}C (μCi/animal)	^{14}C excreted in 24 h (% of dose)	% of 24 h excretion found: Benzoic acid	Hippuric acid	Benzoyl glucuronide	Ornithuric acid
Primates								
Man (2M)	*Homo*	1	5.8	99.4, 99.7	0	100	0	0
Rhesus monkey (3F)	*Macaca*	20	6.8	47 (33–59)	0	100	0	0
Squirrel monkey (2F)	*Saimiri*	50	8.8	46, 49	14, 18	81, 83	5, Trace	0
Capuchin (1F)	*Cebus*	50	8.8	57	0	100	Trace	0
Artiodactyla								
Pig (large white) (2F)	*Suidae*	50	10.1	48, 51	15, 7	85, 93	Trace	0
Lagomorpha								
Rabbit (New Zealand White)	*Leporidae*	49	8.4	60 (36–77)	0	100	0	0
(3F)		200	9.7	86 (79–95)	Trace	98 (97–100)	2 (0–5)	0
Rodents								
Rat (Wistar albino) (3F)	*Muridae*	50	1.0	100 (98–102)	1 (Trace–2)	99 (97–100)	Trace	0
Mouse (I.C.I.) (3×10F)*	*Muridae*	56	0.6	55 (43–67)	Trace	95 (94–96)	5 (4–6)	0
Guinea pig (English) (3F)	*Caviidae*	49	5.0	79 (63–94)	Trace	98 (97–99)	3 (2–3)	0
Hamster (golden) (3F)	*Cricetidae*	52	2.1	99 (98–101)	1	97 (97–98)	1 (1–2)	0
Lemming (steppe) (3F)	*Cricetidae*	56	2.5	98 (94–102)	Trace	100 (99–100)	0	0
Gerbil (3F)	*Cricetidae*	29	3.3	75 (66–82)	2 (0–4)	98 (96–100)	0	0
Carnivora								
Cat (mongrel) (2F)	*Felidae*	51	9.7	29, 86	Trace	100	0	0
Dog (mongrel) (2F, 1M)	*Canidae*	51	8.2	94 (87–100)	0	82 (71–100)	18 (Trace–25)	0
Ferret (mongrel) (3F)	*Mustelidae*	50	7.1	69 (57–90)	9 (6–12)	70 (67–72)	22 (21–22)	0
		198	7.5	78 (62–88)	9 (5–12)	47 (40–54)	44 (41–48)	0
		400	7.5	67 (58–74)	22 (21–23)	30 (24–37)	49 (42–53)	0
Insectivora								
Hedgehog (European) (2M)	*Erinaceidae*	50	6.4	67, 78	5, 7	76, 86	19, 7	0
Chiroptera								
Fruit bat (Indian) (2F)	*Pteropidae*	50	7.8	49, 54	12, 30	Trace	88, 70	0
Birds								
Chicken (Light Sussex) (5F)	*Phasianidae*	50	9.3	56 (38–69)	22 (16–28)	21 (10–43)	3 (0–8)	54 (40–69)
Pigeon (3F)	*Columbidae*	50	6.3	87 (84–102)	15 (13–20)	84 (80–87)	1 (Trace–3)	0
Reptiles								
Turtle (side-necked) (1)†	See footnote	50	6.2	39§	10	16	2	72
Gecko (1×3F)‡	*Gekkonidae*	19	2.5	32‖	3	6	6	85

* The urines of ten animals were pooled for each determination.
‡ The urines of three animals were pooled.
† Also called hidden-necked turtle; sex not known; sub-order Pleurodira.
§ 96 h excretion.
‖ 48 h excretion.

species such as the rabbit (for discussion see Williams, 1959). Table 2 shows that the ferret may be like the dog in this respect, for in the ferret about 22% of the 24h excretion after a dose of 50mg of benzoic acid/kg occurs as benzoyl glucuronide. When the dose is increased to 198mg/kg glycine and glucuronic acid conjugation are about equal, and at 400mg/kg glucuronic acid conjugation is the main mechanism. The ferret also excreted appreciable amounts of free benzoic acid, which may well have arisen by breakdown of benzoyl glucuronide. This effect of dose size in the ferret should be compared with that in the rabbit (see Table 2), an animal that has a relatively high rate of mobilization of glycine for conjugation. At 50mg/kg benzoic acid is excreted in this animal entirely as hippuric acid, and on raising the dose to 200mg/kg glycine conjugation of the acid is still nearly complete (97–100%), only small amounts of benzoyl glucuronide being detectable in the urine.

The hedgehog, an insectivore, has been little used in metabolic studies of this type. Adamson *et al.* (1970) have shown that like many other subprimate species it does not aromatize quinic acid. Other work in this laboratory has shown that it can conjugate phenol with sulphate and glucuronic acid and oxidize phenol to quinol (M. R. French, R. L. Smith & R. T. Williams, unpublished work). Table 2 shows that it converts an oral dose (50mg/kg) of benzoic acid into hippuric acid to the extent of about 80% and also forms benzoyl glucuronide. In this respect it is similar to the dog and ferret.

Table 2 suggests that the Indian fruit bat (*Pteropus giganteus*) may have a defective glycine-conjugation system. Benzoic acid is excreted by this animal entirely as benzoyl glucuronide (70–80% of the 24h excretion) and free benzoic acid. Radiochromatogram scans in solvents *A*, *B* and *C* (Table 1) of fruit-bat urine after dosage with [^{14}C]-benzoic acid showed two peaks corresponding to benzoic acid (minor) and benzoyl glucuronide (major). Reverse isotope dilution for benzoic acid and hippuric acid confirmed that labelled hippuric acid was not present in fruit-bat urine in more than traces. Conjugation with glycine or some other amino acid (e.g. ornithine, arginine etc.; see Smith, 1968) is almost universal among animals, ranging from insects to man. The absence of such a conjugation, however, has been reported in the carrion crow and grey African parrot (Baldwin, Robinson & Williams, 1960) and certain crustacea (Smith, 1968). These observations, however, need confirmation, and the possible absence of hippuric acid synthesis in the fruit bat has now to be investigated at the enzyme level. It is noteworthy that the fruit bat is, like man, unable to synthesize ascorbic acid (Chatterjee, Kar, Ghosh & Guha, 1961; Schmidt-Nielsen, 1967). It does not aromatize quinic acid (Adamson *et al.* 1970), but it conjugates small doses (25mg/kg) of phenol, mainly with glucuronic acid and only to a minor extent with sulphate (M. R. French, R. L. Smith & R. T. Williams, unpublished work).

The findings for the pigeon confirm previous work that this bird converts benzoic acid into hippuric acid and not ornithuric acid (Baldwin *et al.* 1960). With the chicken (Light Sussex) both ornithuric acid and hippuric acid were found. Radiochromatogram scans of chicken excreta after the administration of [^{14}C]benzoic acid showed four radioactive peaks, the largest one being due to ornithuric acid. Although ornithuric acid was the major metabolite, a peak corresponding to hippuric acid was found on the radiochromatogram scans of the excreta of all five hens used. The amount varied from about 5 to 20% of the dose of benzoic acid. In previous work in this laboratory with Brown Leghorn hens (Baldwin *et al.* 1960) it was found that these hens did not form hippuric acid from [^{14}C]benzoic acid, but reverse isotope dilution for hippuric acid in the presence of ornithuric acid is not entirely satisfactory. That glycine conjugation occurs in hens to a minor extent has been reported by Suga (1919) and Chang & Johnson (1956). Both the hen and pigeon excrete appreciable amounts of free benzoic acid (15–20% of 24h excretion), but this could well have arisen from benzoyl glucuronide, which is also present in small amounts in their excreta.

The last two species in Table 2, the turtle and the gecko, excrete the administered [^{14}C]benzoic acid slowly. In both cases, however, the main radioactive metabolite is ornithuric acid, but on the radiochromatogram scans a small peak corresponding to hippuric acid was found. Komori, Sendju, Sagara & Takamatsu (1926) have reported that the turtle excretes hippuric acid after being given benzoic acid, and Khalil (1947) has found hippuric acid in the normal urine of the sea-turtle (*Chelone mydas*), but neither of these papers reported ornithuric acid. Table 2 shows that ornithuric acid (28% of the dose in 96h) is the main metabolite of benzoic acid in the side-necked turtle (species unidentified), although a small amount (6% of the dose) of hippuric acid appears to be present also.

As far as we are aware, the gecko (*Gekko gecko*) has not been previously investigated. It converts benzoic acid mainly into ornithuric acid (27% of the dose in 48h) together with a small amount of what may be hippuric acid (about 2% of the dose) (Table 2). Smith (1958) has found that the green lizard (*Lacerta viridis*) converted benzoic acid into ornithuric acid, but hippuric acid was not detected. Both the gecko and green lizard belong to the reptile sub-order Lacertilia (or Sauria), but to

different infra-orders, namely Gekkota and Scincomorpha respectively.

This work was supported by a grant from the Salsbury Laboratories, Charles City, Iowa, U.S.A.

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J. Pharmacol. 117:39-51, 1956.

ACTION OF SODIUM SALICYLATE AND RELATED COMPOUNDS ON TISSUE METABOLISM *IN VITRO*[1,2]

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Received for publication November 28, 1955

Several salicylates have been studied for their effect on tissue slice respiration. Some investigators (Fishgold *et al.*, 1951; Levy, 1946) have found stimulation of respiration with these agents, while others (Alwall, 1939; Lutwak-Mann, 1942) have reported that salicylates depress this function. Recently, Sproull (1954a) has shown that low concentrations of sodium salicylate (3.5×10^{-4} *M* to 2×10^{-3} *M*) increase oxygen consumption of mouse liver slices while higher concentrations (3×10^{-3} *M* to 7.5×10^{-3} *M*) depress the QO_2 below that of controls. The author placed emphasis on the similarity of the action of the salicylates to that of dinitrophenol.

In the present study it will be shown that the *in vitro* action of the salicylates is qualitatively similar to that of 2,4-dinitrophenol, a compound that uncouples oxidative phosphorylation in mitochondrial preparations, stimulates the respiration of an acceptor-deficient system, inhibits fatty acid oxidation and stimulates the hydrolysis of adenosine triphosphate. Furthermore, increased respiration of certain rat tissues excised from salicylate-treated animals is demonstrated. It is proposed that these actions of the salicylates are responsible for the manifestations of salicylate poisoning.

METHODS. Procedures for the preparation of mitochondria from various tissues and the measurement of their oxidative and phosphorylative activities have been previously described (Brody and Bain, 1951, 1952). Kidney mitochondria were prepared by the method described for liver. Components and conditions of the experiments are described under the respective tables and figures.

ATP-ase activity of brain and liver mitochondria was determined using a modification of the method of Hollunger (1955). Mitochondria of appropriate concentration were added to tubes containing 40 micromoles of KCl and 4 micromoles of ATP, pH 7.4, in 0.25 *M* sucrose. The final volume was 1.7 ml. Incubation time was 15 minutes and the temperature was 25°C. Under these conditions the phosphate liberated was proportional to the amount of mitochondria added. The reaction was stopped by the addition of 0.3 ml. of 50 per cent trichloroacetic acid. Inorganic phosphate was determined on a suitable aliquot by the method of Fiske and Subarrow (1925).

Oxidation of fatty acids was measured in a system similar to that of Kennedy and Lehninger (1949). Octanoate was the substrate with malate as the "spark". Cycling was prevented by the addition of malonate.

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[1] Preliminary reports of this study have been presented at the Fall Meetings of the American Society for Pharmacology and Experimental Therapeutics, Charlottesville, Va., Sept. 1954 (see THIS JOURNAL, 113: 8, 1955); Iowa City, Ia., Sept., 1955; and Pharmacol. Rev., 7: 335, 1955.

[2] Supported by a grant from the USPHS (B-563).

Studies on the respiration of tissue slices after the administration of sodium salicylate were carried out by a modification of the method of Huston and Martin (1954, 1955). Sodium salicylate, 600 mgm./kgm., was administered to rats which were sacrificed after 1 hour. Slices were prepared from liver and kidney by the method of Deutsch (1936) and diaphragm was excised and cut into appropriate strips. The tissues were then spread on ¾ inch squares of fiber-glass filter paper[3] and moistened slightly with Krebs phosphate slice buffer (Umbreit *et al.*, 1949). The filter paper was placed into the center compartment of respirometer flasks in such a manner as to prevent the contact of the tissue with any glass surface. This was necessary for the complete oxygenation of the slice. The center compartment contained no medium. Two-tenths of a ml. of 2*N* KOH was placed in the center well and the same amount of distilled water in the side-arm. The latter addition was made to maintain a constant water vapor pressure. Each flask contained from 60–80 mgm. of tissue. Oxygen consumption was determined by the conventional manometric method. The gas phase was oxygen and the temperature was 37.5°C. Readings were taken at 10 minute intervals and the QO_2's calculated for treated and control animals on the basis of the oxygen uptake values for the first 30 to 40 minutes. Respiration was linear during this period. After 40 minutes the oxygen consumption dropped to about eighty per cent of the initial values. At the end of the incubation period the slices were removed from the flasks and weighed. The QO_2 was calculated on a wet weight basis. As has been shown by Huston and Martin (1954), the respiratory rate of tissues in contact with oxygen on fiber-glass filter paper is satisfactorily maintained. The decline in oxygen consumption with time is minimal and the respiratory rates obtained by this method are as high or higher than those for tissue slices run in fluid media.

RESULTS: *Uncoupling in liver and kidney mitochondrial preparations.* The similarity of the action of sodium salicylate and sodium acetylsalicylate to that of 2,4-dinitrophenol (DNP) can be observed by comparing the effects of these agents on mitochondrial preparations. The results of sodium salicylate and sodium acetylsalicylate addition to rat liver mitochondrial systems are shown in figure 1. The data indicated were calculated as per cent of control activity. Oxygen uptake and inorganic phosphate uptake were determined for 20 to 30 minute incubation periods, the time of incubation being dependent upon the activity of the preparation. P:O values were calculated from these values. (The P:O ratio is the ratio of the micromoles of inorganic phosphate disappearing from the medium to the microatoms of oxygen consumed.) In the experiments shown in figure 1 pyruvate plus malate was used as the substrate, but comparable results could also be obtained with succinate, glutamate, α-ketoglutarate or oxalacetate. It may be observed that the effect of these salicylates on oxygen consumption is minimal; at 2×10^{-3} *M* these agents depress oxygen consumption by about twenty per cent. At this same drug concentration, sodium salicylate completely inhibits the uptake of inorganic phosphate (P:O = 0). Sodium acetylsalicylate is slightly less potent than sodium salicylate.

A limited number of experiments indicate that rat kidney mitochondria resemble rat liver mitochondria in their sensitivity to salicylates. With pyruvate plus malate, succinate, α-ketoglutarate or malate as the substrate, the addition of 2×10^{-3} *M* sodium salicylate depresses oxygen consumption of kidney mitochondria by 15–25 per cent and inorganic phosphate by 70–90 per cent.

Uncoupling in brain mitochondrial preparations. Figure 2 illustrates the effect

[3] H. Reeve Angel and Co., New York, N. Y.

of the same two agents on oxidative phosphorylation in rat brain mitochondria. In brain, the inhibition of phosphorylation by the salicylates is less pronounced than in liver. In addition there is a definite increase in oxygen consumption in the presence of these agents. While this increase from control levels is variable (mean 18 per cent, range 0–37 per cent), in 30 experiments the addition of sodium salicylate or sodium acetylsalicylate failed to depress oxygen consumption. Dinitrophenol, in concentrations that completely inhibit phosphorylation (2.5×10^{-5} *M*) depresses rat brain mitochondrial oxidation by 40 per cent with pyruvate plus malate as the substrate (Brody and Bain, 1954). The stimulatory effect on oxygen consumption with the salicylates was seen only when pyruvate plus malate was used as substrate. In the presence of glutamate, succinate or α-ketoglutarate, 2×10^{-3} *M* sodium salicylate depressed oxygen consumption by 20 to 30 per cent and inorganic phosphate uptake by 50 to 70 per cent. Sodium salicylate had no effect on the hexokinase reaction in the concentrations used in these studies.

The stimulation of brain mitochondrial respiration by salicylates was an unexpected result, since the enzymes are saturated with both inorganic phosphate and acceptor. The possibility existed that the salicylates might be binding endogenous calcium ion as calcium is known to depress mitochondrial respiration

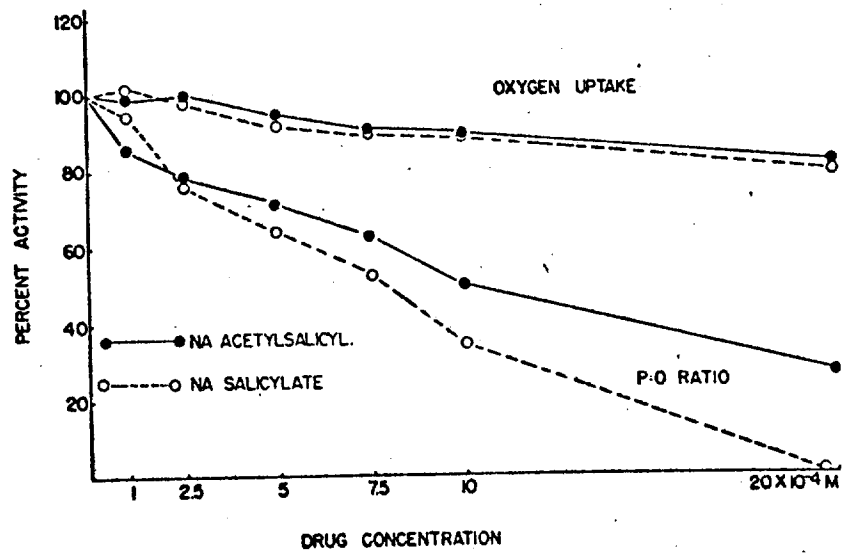

FIG. 1. The effect of sodium salicylate and sodium acetylsalicylate on oxidative phosphorylation in rat liver mitochondria. Reaction mixture: 1.7×10^{-2} *M* K-phosphate buffer, pH 7.4, 1.7×10^{-2} *M* KCl, 1.3×10^{-2} *M* $MgCl_2$, 2.8×10^{-3} *M* K-ATP, 1.5×10^{-3} *M* Na-pyruvate, 2.0×10^{-3} *M* Na-malate, 1.0×10^{-5} *M* cytochrome C, 30 units yeast hexokinase, 3.3×10^{-2} *M* glucose, drug concentrations as indicated, rat liver mitochondria containing 0.7–1.0 mgm. total nitrogen and 1.5×10^{-2} *M* NaF. Total volume 1.8 ml. The sidearm contained 0.2 ml. 50 per cent trichloroacetic acid and the center well 0.2 ml. 2*N* KOH. Components were added in the order indicated. The temperature was 25°C. and the gas phase air. Each point in the figure is the average of at least 6 separate experiments.

(Slater and Cleland, 1952). To test this hypothesis, brain mitochondria were prepared in 0.25 M sucrose to which was added 1×10^{-3} M disodium ethylenediaminetetraacetate (disodium versenate). The results of such an experiment are shown in table 1. Oxidation and phosphorylation in brain mitochondria prepared with disodium versenate are compared with the activity of mitochondria prepared only in 0.25 M sucrose. The data indicate that versene failed to influence the stimulation of oxygen consumption of brain mitochondria by sodium salicylate.

Stimulation of an acceptor-deficient system. A confirmatory method for determining uncoupling makes use of the so-called "acceptor-deficient" system. The effect of acceptor addition on the rate of mitochondrial oxidation is well known (Lardy and Wellman, 1952). These workers have shown that the respiratory rate may be stimulated by the addition of an acceptor system such as the "glucose-hexokinase" trap. Since oxidation and phosphorylation are coupled, the rate of oxidation depends upon the removal or transfer of ATP. Ordinarily, in a system saturated with acceptor an uncoupling agent will not stimulate the initial respiratory rate. The rate in an acceptor-deficient system with an uncoupling agent will parallel the rate of an acceptor-saturated mitochondrial system without uncoupler because the uncoupling agent removes the requirement for acceptor. The effect of sodium salicylate and dinitrophenol on an acceptor-deficient rat liver mitochondrial system is shown in figure 3. It may be observed that 1×10^{-5} M dinitrophenol and 4×10^{-4} M sodium salicylate eliminate the acceptor requirement. The rates obtained with these agents but without the

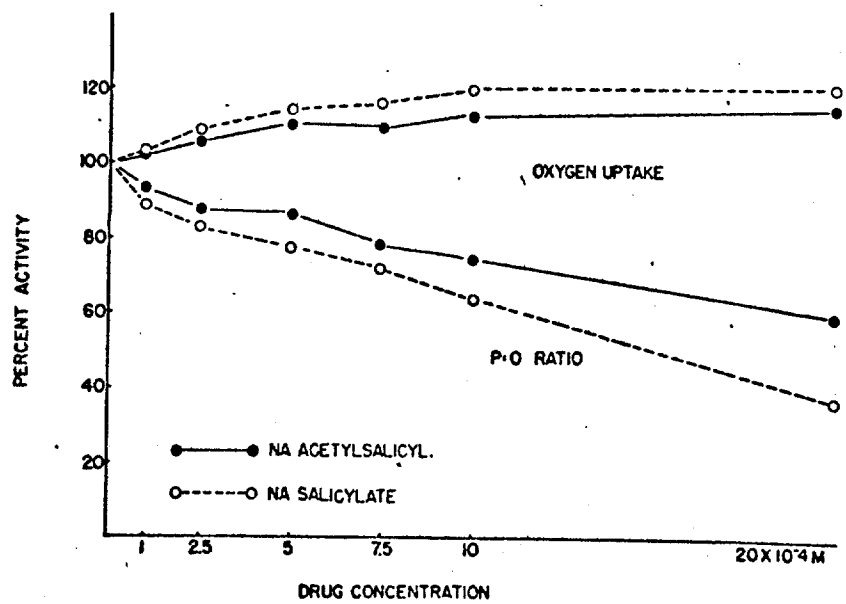

FIG. 2. The effect of sodium salicylate and sodium acetylsalicylate on oxidative phosphorylation in rat brain mitochondria. Conditions same as figure 1. Brain mitochondrial preparations contained 1.2–1.5 mgm. total nitrogen. Each point is the average of at least 6 separate experiments.

TABLE I

The failure of disodium versenate to reverse sodium salicylate stimulation of brain mitochondrial respiration. Experimental conditions same as figure 2

Drug	Molar Conc.	O_2*	IP†	P:O
Mitochondria in 0.25 *M* sucrose				
Control	—	7.25	14.4	1.98
Control	—	6.82	12.0	1.76
Sodium salicylate	2×10^{-3}	9.86	5.6	0.57
Sodium salicylate	2×10^{-3}	9.35	8.8	0.94
Mitochondria in 0.25 *M* sucrose and 0.001 *M* disodium versenate				
Control	—	8.36	20.0	2.39
Control	—	7.97	18.4	2.31
Sodium salicylate	2×10^{-3}	9.80	8.8	0.90
Sodium salicylate	2×10^{-3}	10.40	8.8	0.84

* Microatoms of oxygen uptake.
† Micromoles of inorganic phosphate uptake.

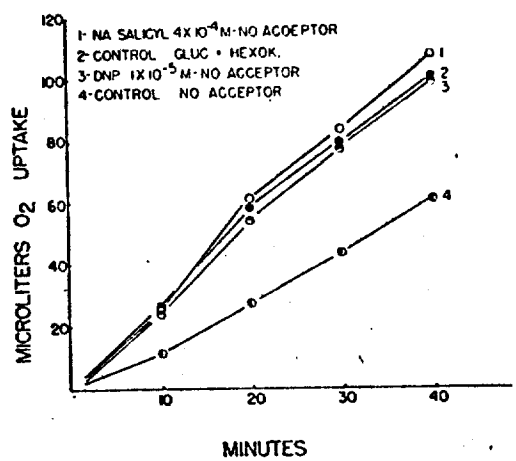

FIG. 3. Effect of dinitrophenol and sodium salicylate on oxygen uptake in an acceptor-deficient system. Conditions same as figure 1 except as indicated. Rat liver mitochondria, total nitrogen per flask 1.0 mgm.

glucose-hexokinase trap are equal to the control rate of the mitochondrial system saturated with glucose and hexokinase.

Inhibition of fatty acid oxidation. The fatty acid oxidase system of liver is sensitive to such uncoupling agents as dinitrophenol, the barbiturates and gramicidin. Every effective uncoupling agent tested has proved to be a potent inhibitor of fatty acid oxidation (Kennedy and Lehninger, 1952). The effect of salicylates on fatty acid oxidation of rat liver mitochondria is shown in figure 4. The results are graphed as the per cent of control activity. Octanoate oxidation is markedly inhibited by low concentrations of both sodium salicylate and sodium acetylsali-

cylate. At a concentration of 1×10^{-3} *M* these compounds depress fatty acid oxidation by about 60 per cent.

Stimulation of hydrolysis of ATP by salicylates. Several nitro and halophenols when added to mitochondria enhance the liberation of inorganic phosphate from ATP (Hunter, 1951; Potter and Recknagel, 1951). It has been suggested that the uncoupling action of dinitrophenol and the stimulatory effect on ATP hydrolysis are related, although the relationship is obscure at this time. Recent studies have indicated that DNP uncouples oxidative phosphorylation by a mechanism other than the enhancement of ATP-ase activity (Cohn, 1953). The effect of sodium salicylate and DNP on ATP-ase activity of mitochondria was compared (table 2). Sodium salicylate like dinitrophenol does stimulate ATP breakdown. The concentrations needed for this hydrolysis are larger than those which uncouple oxidative phosphorylation. Using the system described, stimulation of ATP-ase activity could always be demonstrated with sodium salicylate in liver mitochondrial preparations and in most brain mitochondrial preparations. Incubation with versene, found essential by other workers to show enhancement of ATP-ase in brain mitochondria with other agents (Maxwell and Nickel, 1954; Bain, 1955), was not necessary in the present studies.

Failure of the magnesium ion to reverse salicylate uncoupling. Kaplan *et al.* (1954) have shown that salicylates inhibit Krebs cycle oxidations in kidney and liver homogenates. They reported that this inhibition could be reversed by an excess of magnesium ions. These effects were observed using 6.7×10^{-3} *M*

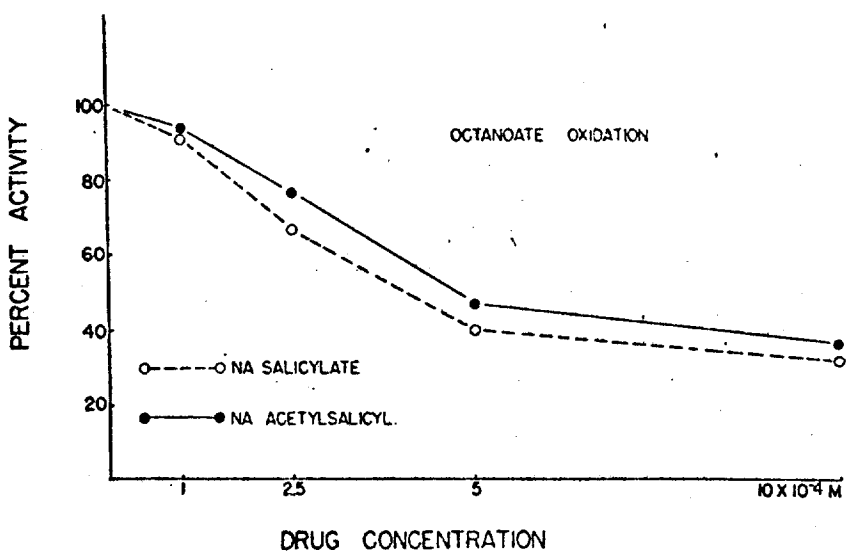

FIG. 4. Effect of salicylates on fatty acid oxidation. Reaction medium: 1.6×10^{-2} *M* Na-glycylglycine buffer pH 7.4, 2.2×10^{-2} *M* Na-phosphate buffer pH 7.4, 1.6×10^{-2} *M* KCl, 1.3×10^{-2} *M* $MgCl_2$, 2.8×10^{-3} *M* K-ATP, 1.0×10^{-5} *M* cytochrome C, 2.0×10^{-3} *M* K-octanoate, 5.0×10^{-4} *M* K-malate, 3×10^{-3} *M* Na-malonate, drug concentrations as indicated, rat liver mitochondria containing 1.2-1.5 mgm. total nitrogen in a volume of 1.8 ml. Two-tenths of 2*N* KOH in center well, gas phase air, incubation time 30-40 minutes, temperature 25°C. Each point represents the average of 3 separate experiments.

TABLE 2

The effect of sodium salicylate and dinitrophenol on brain and liver mitochondrial ATP-ase activity. Conditions as described in the text

Drug	Molar Conc.	Micromoles IP Liberated	
		Brain*	Liver†
Control	--	0.37	0.48
Control	—	0.41	0.40
Sodium salicylate	5×10^{-3}	1.73	1.08
Sodium salicylate	2.5×10^{-3}	0.57	0.74
2,4-Dinitrophenol	1×10^{-4}	1.29	1.86
2,4-Dinitrophenol	5×10^{-5}	0.71	1.28

* 0.1 ml. brain mitochondria equivalent to 100 mgm. tissue.
† 0.025 ml. liver mitochondria equivalent to 25 mgm. tissue.

TABLE 3

Effect of magnesium on salicylate inhibition of oxidative phosphorylation. Conditions same as for figure 2. Incubation time 30 minutes. Pyruvate plus malate substrate.

Magnesium Concentration, micromoles/ml.	Control			2×10^{-3} Sodium Salicylate			% Uncoupling
	IP*	O_2†	P:O	IP*	O_2†	P:O	
3	17.6	8.5	2.07	7.2	9.5	0.76	63
6	20.8	9.1	2.28	13.6	10.0	1.36	40
12	22.4	9.8	2.28	12.0	11.1	1.08	53
24	22.4	9.5	2.36	10.2	10.5	0.96	59

* Micromoles of inorganic phosphate uptake.
† Microatoms of oxygen uptake.

sodium salicylate. It has also been shown that the inhibition of oxidative phosphorylation by the tetracycline antibiotics can be reversed by the addition of magnesium (Brody *et al.*, 1954). The result of excess magnesium addition on oxidation and phosphorylation in a rat brain mitochondrial preparation is shown in table 3. Neither the rate of oxidation nor the incorporation of inorganic phosphate in the presence of salicylate seems to be affected by altering the magnesium concentration of the reaction medium. It should also be noted that 2×10^{-3} *M* salicylate stimulates the rate of oxygen uptake of brain mitochondria. Excess magnesium does not reverse the inhibition of oxidative phosphorylation produced by dinitrophenol (Brody and Bain, 1954).

Uncoupling action of compounds related to the salicylates. If the dissociation of oxidative phosphorylation is in some way concerned with the analgesic, antipyretic or antirheumatic properties of the salicylates, then pharmacologically-related compounds might also be uncoupling agents. Three classes of compounds were tested and compared with the salicylates: antipyretics chemically unrelated to the salicylates, antirheumatic agents, and a miscellaneous group of compounds chemically related to the salicylates. The results of these studies are shown in table 4. All of the compounds assayed were run over a wide spectrum of concentrations. The experiments in table 4 are typical and have been selected to

TABLE 4

Effect of some compounds related to the salicylates on the uncoupling of oxidative phosphorylation

Exp.	Drug or Control	O_2*	IP†	P:O	Average P:O	% Uncoupling
1	Control	7.82	22.4	2.86	2.97	
		6.73	20.8	3.08		
	$2 \times 10^{-3}M$ sod. salicylate (2-hydroxybenzoate)	6.12	0.8	0.13	0.35	96
		5.66	3.2	0.56		
	$2 \times 10^{-3}M$ beta-resorcylate[4] (2,4-dihydroxybenzoate)	5.87	16.4	2.80	2.69	9
		5.75	14.8	2.58		
	$2 \times 10^{-3}M$ gamma-resorcylate[5] (2,6-dihydroxybenzoate)	6.62	16.4	2.48	2.64	10
		5.86	16.4	2.80		
2	Control	7.95	24.8	3.11	3.05	
		7.52	22.4	2.98		
	$2 \times 10^{-3}M$ ortho-pyrocatechuate[4] (2,3-dihydroxybenzoate)	6.44	10.4	1.62	1.35	55
		5.88	6.4	1.09		
3	Control	7.95	23.2	2.92	2.84	
		8.73	24.0	2.75		
	$2 \times 10^{-3}M$ sod. benzoate	7.25	20.8	2.87	2.86	0
		7.60	21.6	2.84		
4	Control	10.80	28.0	2.59	2.55	
		10.50	26.4	2.51		
	$2 \times 10^{-3}M$ PAS (4-amino, 2-hydroxybenzoate)	9.59	24.0	2.50	2.47	3
		10.20	24.8	2.43		
5	Control	8.35	20.8	2.49	2.44	
		8.37	20.0	2.39		
	$2 \times 10^{-3}M$ sod. gentisate[4] (2,5-dihydroxybenzoate)	8.91	20.8	2.33	2.27	7
		8.35	18.4	2.21		
	$2 \times 10^{-3}M$ salicylamide[4] (2-hydroxybenzamide)	8.40	17.6	2.10	2.16	11
		10.20	22.4	2.20		
6	Control	10.8	26.4	2.43	2.48	
		10.4	26.4	2.53		
	$2 \times 10^{-3}M$ sod. salicylate	8.87	0.8	0.04		99
	$2 \times 10^{-3}M$ sod. acetylsalicylate (2-acetoxybenzoate)	8.17	5.6	0.69		72
7	Control	7.95	24.8	3.20	3.20	
		7.48	24.0	3.2		
	$2 \times 10^{-3}M$ antipyrine	6.24	22.4	3.59	3.32	0
		7.19	22.0	3.06		
	$2 \times 10^{-3}M$ acetophenetidin	6.72	20.8	3.10	3.07	4
		8.50	25.6	3.04		
8	Control	6.97	20.0	2.87	2.62	
		6.85	17.6	2.57		
	$2 \times 10^{-3}M$ methyl salicylate	4.65	3.2	0.69	0.69	74

* Microatoms of oxygen uptake.

† Micromoles of inorganic phosphate uptake.

show comparative uncoupling activity at a single drug concentration. Rat liver mitochondria were the enzyme source in each experiment.

[4] Kindly supplied by Dr. P. K. Smith, George Washington University.

[5] Kindly supplied by Dr. Lathan Crandall, Miles-Ames Laboratories.

Of the compounds assayed, sodium salicylate, aspirin and methyl salicylate were the most potent, while o-pyrocatechuate was somewhat less effective in uncoupling phosphorylation. The magnitude of the inhibition of phosphorylation by methyl salicylate was comparable to that obtained with acetylsalicylate, although methyl salicylate appeared to have a greater depressant effect on oxidation. All of the other drugs studied had very weak or no uncoupling action. Especially interesting are the results obtained with the dihydroxy derivatives of benzoic acid. The 2,3-dihydroxy compound (o-pyrocatechuate) had about 50 per cent of the activity of sodium salicylate. The 2,4-dihydroxybenzoate (β-resorcylate), 2,5-dihydroxybenzoate (gentisate) and the 2,6-dihydroxybenzoate (γ-resorcylate) were practically devoid of uncoupling activity. Both gentisate and γ-resorcylate are effective antirheumatic agents. Para-aminosalicylate (PAS) which will reverse the stimulation of oxygen consumption produced by sodium salicylate in tubercle bacilli (Bernheim, 1940; Lutz, 1949), and is an effective tuberculostat, does not inhibit oxidation or phosphorylation in liver mitochondria. Salicylamide, acetophenetidin, antipyrine and sodium benzoate are also ineffective as uncouplers.

Respiration of tissue slices from salicylate-treated animals. The effect of sodium salicylate on the oxygen consumption of diaphragm, liver and kidney was examined using the fiber-glass filter paper technic. The results of such experiments are shown in table 5. Significance was determined by use of the Student "t" test as described by Snedecor (1946). At 600 mgm./kgm. intraperitoneally, the rate of oxygen uptake of diaphragm strips and liver slices in the salicylate-treated animals was significantly elevated when compared with rates from control animals. The respiratory rate of kidney slices from treated animals was the same as the controls. To determine whether or not the failure of the kidney to respond was a function of the dose administered, animals were also treated with 400 and 800 mgm./kgm. of sodium salicylate. At these concentrations again no stimulation of oxygen consumption of kidney was observed. No explanation for these findings is apparent at the present time. Brain slice respiration was not studied for several reasons. Only about one-third to one-fourth as much sodium salicylate appears in the brain as in serum or in other tissues (Smith, 1949). We have shown that the peripheral tissues are more sensitive than is brain to the uncoupling action (figures 1, 2). It would appear, therefore, that very much larger doses of

TABLE 5

Respiration of some rat tissues on fiberglass filter paper after administration of 600 mgm./kgm. sodium salicylate

Organ	Controls			Treated			Significance "p"
	No. expts.	Mean QO_2*	St. dev.	No. expts.	Mean QO_2*	St. dev.	
Diaphragm	25	1.71	0.25	19	2.92	0.60	.01
Liver	26	2.59	0.37	18	3.62	0.64	.01
Kidney	22	4.71	0.78	18	5.01	1.0	NS

* QO_2 = microliters oxygen uptake per hour per milligram tissue wet weight.

sodium salicylate would be needed to produce an effect comparable to that seen on the liver, for example. Furthermore, we have shown that the salicylates may stimulate brain respiration in some manner unrelated to the uncoupling phenomenon (figure 2). Thus, a positive result using the fiber-glass method on brain would not necessarily be an indication of *in vivo* uncoupling activity.

DISCUSSION: We have found that the salicylates will uncouple oxidative phosphorylation in a manner similar to dinitrophenol, although the latter compound is considerably more potent. There are differences that deserve further comment. One of the most interesting observations is that the peripheral tissues, liver and kidney, are more sensitive to the salicylates than is brain. The salicylates appear to be unique in this respect. With other uncoupling agents studied, brain has proved to be more sensitive than other tissues (Brody and Bain, 1954). While it is true that sodium salicylate appears in the brain in smaller amounts than in other tissues after an administered dose, one would not expect to find brain any less responsive in mitochondrial preparations where the cell barrier is non-existent.

There is another striking difference between brain and liver mitochondria when salicylates are added *in vitro*. This is the stimulation of oxygen uptake observed when pyruvate plus malate is used as the substrate. This stimulatory effect on the respiratory rate of brain mitochondria is not the result of the uncoupling action of the salicylates since the system is saturated with both inorganic phosphate and acceptor. Nor is stimulation observed when substrates other than pyruvate are employed. Further, this increase in rate is not seen when salicylates are added to either liver or kidney mitochondria. Our experiments have ruled out a calcium-binding effect of the salicylates as being implicated in the stimulation with pyruvate. However, we have not discounted the possibility that these compounds combine with or inhibit some rate-controlling enzyme or cofactor in the brain mitochondrial system. While the stimulation of oxygen consumption is not consistent, in no experiment has sodium salicylate or aspirin actually depressed oxygen consumption. The data presented here are in contrast to the findings that pyruvate oxidation is one of the most sensitive steps in the citric acid cycle, while succinate oxidation, on the other hand, is ordinarily resistant to many agents (Persky *et al.*, 1950; Quastel, 1953; Gordon and Elliott, 1947). Our findings confirm the work of Patel and Heim (1954) who have found that high concentrations of sodium salicylate stimulate pyruvate oxidation in rat brain homogenates by 60 per cent.

In evaluating and interpreting our findings we are making no attempt to relate the effects observed *in vitro* with the specific pharmacologic actions of the salicylates such as antipyresis, analgesia or antirheumatic action. Rather, we will point out that many of the toxic symptoms associated with salicylate overdosage could be attributed to the ability of this class of compounds to uncouple oxidative phosphorylation. The toxicity seen with most pharmacological agents is an extension of their therapeutic action. This situation apparently does not hold true for the salicylates. Many of the signs of toxicity with the salicylates parallel those observed after the administration of dinitrophenol. It is well known that in large

doses salicylates like DNP produce a hyperthermia in man as well as other species (Smith, 1949; Gross and Greenberg, 1948; McGuigan and Higgins, 1935). In man and in the experimental animal, sodium salicylate and aspirin will increase oxygen consumption (Cochran, 1952; Balogh *et al.*, 1952). Cochran found that the oral administration of large amounts of acetylsalicylic acid to man caused a marked increase in oxygen consumption and a fall in the respiratory quotient. This would be the expected response if the salicylates induce uncoupling and the energy derived from carbohydrate breakdown were insufficient to meet the normal demands of the body. The organism would then be compelled to utilize fat and protein as energy sources to make up the deficit. The salicylates also deplete liver glycogen (Lutwak-Mann, 1942; Sproull, 1954) and most workers have found that large doses of salicylates will cause hyperglycemia and glycosuria (Gross and Greenberg, 1948). These findings would also point to an increased demand for energy by the animal. A similar metabolic pattern is reported for dinitrophenol (von Oettingen, 1949).

Several other signs of salicylate intoxication are worthy of comment. These compounds produce a hyperpnea which is believed to be central in origin. This change in the character of the respiration leads to an alkalosis which later develops into a ketonic acidosis. Why a salicylate intoxicated animal should become acidotic is not well understood. The acidosis does not appear to be related to the base-binding properties of the salicylates *per se*. From our *in vitro* studies it would appear that the salicylate uncoupling could very well result in increased fat and protein utilization, or gluconeogenesis. If in addition fatty acid oxidation were also partially depressed (figure 4) then one would expect a ketosis in a salicylate-intoxicated animal. If high-energy bond compound generation were also sufficiently inhibited the synthesis of protein would also be influenced. Perhaps the hypoprothrombinemia associated with salicylate overdosage is the result of such an effect in the liver.

Studies with compounds chemically and pharmacologically related to the salicylates would also tend to validate the hypothesis that the salicylates produce their toxicity by uncoupling oxidative phosphorylation. Certainly, overdosage with antipyrine or acetophenetidin produces none of the symptoms in the intact animal described for salicylates. Salicylamide is considerably more depressant than sodium salicylate or acetylsalicylic acid, but is of lower toxicity and has none of the toxic signs commonly seen with the latter agents (Litter *et al.*, 1951). Gentisic acid apparently does not deplete liver glycogen or produce an acidosis and is less toxic than salicylate (Shaefer *et al.*, 1950; Clarke *et al.*, 1953). The pharmacology of ortho-pyrocatechuate has not been well studied. From our data it would also appear that the uncoupling obtained with the salicylates is not related to antirheumatic activity. Both gentisate and γ-resorcylate are poor uncoupling agents when compared to sodium salicylate, yet apparently are effective in the treatment of rheumatic fever. Meade (1954) has observed that salicylate increased the oxygen consumption of rats by 80 per cent, while benzoate, p-aminobenzoate, and the 2-3, 2-4, 2-5, 2-6, and 3-4, dihydroxybenzoates had no effect on oxygen consumption.

The uncoupling potency of sodium salicylate is somewhat greater than either aspirin or methyl salicylate. The difference between acetylsalicylate and methyl salicylate and sodium salicylate could be accounted for if one invokes the necessity of the hydrolysis of the esters to salicylate before an effect may be observed.

SUMMARY

Sodium salicylate, aspirin and methyl salicylate uncouple oxidative phosphorylation in liver, kidney and brain mitochondrial preparations. Liver and kidney are more sensitive to these agents than is brain. The uncoupling action is qualitatively similar to that seen with the classical uncoupling agent, 2,4-dinitrophenol. The concentrations of salicylates used in these studies are comparable to toxic levels in the intact animal.

These compounds stimulate the oxygen consumption of brain mitochondria in an ortho phosphate and acceptor-saturated system, with pyruvate and malate as substrate. This effect is not abolished by incubation of brain mitochondria with ethylenediaminetetraacetate.

Salicylates stimulate the respiratory rate of an acceptor-deficient system and inhibit fatty acid oxidation of rat liver mitochondria. Like dinitrophenol, these agents enhance the liberation of inorganic phosphate from ATP.

Sodium salicylate, aspirin, methyl salicylate, and o-pyrocatechuate effectively depress oxidative phosphorylation while sodium benzoate, gentisate, β-resorcylate, γ-resorcylate, para-aminosalicylate, salicylamide, antipyrine and acetophenetidin are weak or ineffective uncoupling agents.

The oxygen consumption of liver and muscle slices from salicylate treated-rats is increased.

It is suggested that the above actions are responsible for many of the manifestations of salicylate poisoning.

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INCREASING AND PROLONGING BLOOD PENICILLIN CONCENTRATIONS FOLLOWING INTRAMUSCULAR ADMINISTRATION[1]

Two general methods have been employed in maintaining effective blood levels of penicillin following intramuscular administration; those which delay the absorption of a large dose and those which delay excretion of penicillin. Delayed absorption has been accomplished by giving the penicillin in oil,[2] in beeswax-peanut oil[3] mixtures, in gelatin,[4] by local ice packs[5] and with a vasoconstrictor such as adrenalin.[6] Delayed excretion has been effected by the simultaneous administration of diodrast,[7] para-aminohippuric acid[8, 9] and has been observed in subjects with renal failure.[10] Penicillin administered under these conditions may result in a blood level two to four times as high as that obtained by intramuscular injection every 2 to 4 hours. Although 0.03–0.06 units per cc of serum obtained by conventional methods have been considered to be therapeutically effective,[11, 12, 13] this range of concentration does not exceed the sensitivity level of many of the commonly encountered pathogenic micro-organisms. Furthermore, in certain chronic diseases such as subacute bacterial endocarditis and osteomyelitis it is desirable to maintain higher levels over long periods of time. Since large amounts of penicillin and technical difficulties in administration are factors in obtaining high blood levels, a simple means of obtaining increased blood levels on lower dosages should be useful.

Following the observation that para-aminohippuric acid competes with penicillin for excretion in renal tubules[8, 9] as has been suggested previously for diodrast and related compounds,[7] the writers have used benzoic acid administered orally to accomplish the same purpose. Benzoic acid is detoxified in the normal liver by conjugation with glycine to form hippuric acid. Sodium benzoate has had extensive use in the treatment of rheumatic fever when salicylates can not be used. Daily doses of 12 gm over long periods of time and as much as 50 gm during 1 day have been given without untoward effects.[13] The penicillin blocking power of benzoic acid, however, may be small on an unrestricted diet. For this reason, fluid and salt restrictions have been combined with benzoic acid therapy (Table 1).

TABLE 1

	G.McL. (I)	M.L. (II)	W.G. (I)	J.M. (I)	W.J. (III)	P.DeG. (II)
Penicillin dosage	20,000 u. q 2h	10,000 u. q h	20,000 u. q 2h	20,000 u. q 2h	20,000 u. q 3h	20,000 u. q 3h
Penicillin with normal fluids and normal diet						
30 min. level	0.14	0.14	0.14	0.28	0.28	
60 " "	0.07	0.07	0.07	0.14	0.07	
90 " "	0.07	...	0.07	0.07	trace	
120 " "	trace	...	trace	trace	0.0	
180 " "	...	...	...	...	0.0	
2 hr. urine exer.	56%	...	...	...	54.4%	
Penicillin with low fluids and low salt diet						
30 min. level	0.28	...	0.28	0.56	0.28	0.28
60 " "	0.14	...	0.21	0.28	...	0.11
90 " "	...	...	...	...	0.14	...
120 " "	0.07	...	0.11	0.11	...	trace
180 " "	0.0	...	...	...	trace	...
2 hr. urine exer.	50.4%	...	...	...	48.8%	...
Penicillin with normal fluids and normal diet plus benzoic acid						
30 min. level	0.28	0.28	0.56	0.56	0.28	...
60 " "	0.14	0.14	0.28	0.28	...	...
90 " "	...	...	...	...	0.14	...
120 " "	0.07	...	0.14	0.14	...	...
180 " "	trace	...	...	...	0.0	...
2 hr. urine exer.	46%	...	...	...	49.1%	...
Penicillin with low fluids and low salt diet plus benzoic acid						
30 min. level	1.12	...	1.12	1.12	0.56	0.56
60 " "	0.56	...	0.56	0.56	0.28	0.28
90 " "	...	...	...	...	0.11	...
120 " "	0.28	...	0.28	0.28	...	0.07
180 " "	0.11	...	...	...	0.0	...
2 hr. urine exer.	22.4%	...	...	...	46%	...

(I) = Subacute bacterial endocarditis.
(II) = Gonococcal arthritis.
(III) = Syphilis.

Methods: Benzoic acid crystals were given as a suspension in a sweet gelatin dessert or in acacia flavored with syrup of orange to prevent local irritation of the throat during ingestion, or in 0.6 gm gelatin capsules. The usual dose was 2.5 gm or 4 capsules every 4 hours. Most patients preferred the capsules. Sodium penicillin 20,000 units in 2 cc of physiological saline was given intramuscularly every 2 or 3 hours. Penicillin levels were determined on the serum of freshly drawn blood which was allowed to clot before centrifugation. The assays were done by the broth dilution tube method of titration using a standard culture of *Staphylococcus aureus*. Control levels following single intramuscular injections of penicillin in saline agreed closely with those for the same doses reported

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by other investigators.[10, 11, 12, 14] During the control period the patients were placed on a daily intake of 1,500 to 2,000 calories with the diet containing 6 to 10 gm of salt and a fluid intake of 2,500 to 3,000 cc. Penicillin levels were determined after the third day of this regimen. During combined treatment the penicillin was given 20 to 30 minutes after the benzoic acid. At first sodium benzoate was tried, but it was found that the added base neutralized the effects of a controlled salt intake, and in addition the dose of sodium benzoate proved to be somewhat nauseating. On the normal diet 2.5 gm of benzoic acid equalled 6 gm of sodium benzoate in raising the serum penicillin level. During diet restrictions which were in effect 3 days before penicillin levels were determined, the patients received the same caloric intake as previously, but fluids were limited to 1,000 to 1,500 cc a day and salt to 3 gm or less a day. On the restricted fluid intake the urine volume commonly fell to 400 to 600 cc in 24 hours.

Six patients have been studied so far in this series. In addition to determining the degree of elevation and effective maintenance of blood levels, urine levels were done to check the per cent. excretion of penicillin in a 2-hour period following intramuscular injection. The experimental results of this phase of the study are presented in Table 1.

Results: The 1-hour peak serum levels using 20,000 units of penicillin alone were in the 0.07 units per cc range. Two-hour serum levels, however, were ineffective. Using the low salt and fluid diet plus benzoic acid 1-hour levels were 0.56 units per cc and 2-hour levels 0.28 units per cc. A fall from 56 to 22 per cent. in the 2-hour urine excretion of penicillin as a result of diet and benzoic acid treatment reflects a similar trend (Chart 1). This represents an eight-fold increase in, as well as a prolongation of, the penicillin blood level. In the work with adrenalin[6] using two and one-half times the dose of penicillin, the 2-hour levels were one fourth this amount, except for one case which showed the same level. With repeated injections every 2 to 3 hours, restriction of salt and water intake and administration of benzoic acid every 4 hours, the minimum levels obtained were 0.14–0.28 units per cc of serum. This is a five to ten-fold increase over the levels usually maintained when two to five times greater doses of penicillin are used. This method of treatment is being applied to penicillin therapy in acute, subacute and chronic conditions, and will be discussed elsewhere.

Summary: (1) Restriction of fluid intake to 1,500 cc and the salt intake to 3 gm a day doubles the penicillin blood level following interrupted intramuscular injections of penicillin. (2) The administration of benzoic acid to a patient on an unrestricted diet may double the penicillin blood level during similar treatment. (3) The combination of these two procedures results in a four- to eight-fold increase in penicillin blood level with a prolonged effective blood concentration.

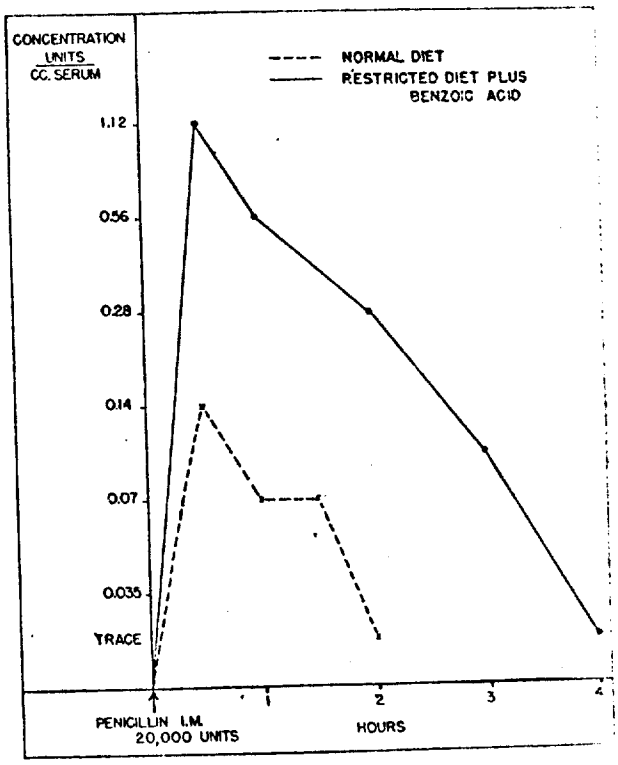


CHART 1

JACK BRONFENBRENNER
CUTTING B. FAVOUR

Arzneimitt.-Forsch. 7(6): 340-345, 1957.

ON THE PHARMACOLOGY OF BENZOIC ACID, OF PARACHLOROBENZOIC ACID AND OF PARAOXYBENZOIC ACID AND ITS ESTERS.

M. v. Bubnoff, D. Schnell and J. Vogt-Moykoff

In a previous communication, pharmacological effects not known before had been reported with respect to several benzoic acid preparations used as preservatives (Bubnoff etal). Besides vascular spasmolytic and ganglioplegic effects, we also were able to show a performance-decreasing effect on the striated musculature pretreated with Prostigmine. Further experiments were supposed to resolve the question whether pharmacological effects can be shown for oral administration of such substances. So far it is only known for benzoic acid that anticonvulsive effects will occur even for oral ingestion, -Zwissler. According to Schuebel & Manger and Metzger etal, the paraoxybenzoic acid esters however are completely reabsorbed from the intestine whether saponified or not. Sokol was able to show non-saponified esters in the plasma (also see Richardson and collaborators) Therefore one had to expect that oral ingestion of paraoxybenzoic acid esters would also show pharmacological effects. The methods we had previously used seemed unsuited for testing orally administered substances which might not become effective until after lengthy reabsorption processes had taken place. Therefore we made use of the method of Eichholtz & Hoppe for testing the anticonvulsive effects in the cocain cramps in rats; this is the method also used by Zwissler for benzoic acid. Following a check of Zwissler's results, such tests were supposed to be carried out also with respect to parachlorobenzoic acid and parachlorobenzoic acid esters.

Furthermore, carrying on with prior research concerning the vascular spasmolytic effects in the kidneys and extremities and also as regards anticonvulsive effectivenss, the influence of benzoic acid and its derivatives on brain blood supply was examined.

METHODOLOGY

We used the method of Eichholtz & Hoppe for testing the anticonvulsive effects of benzoic acid, parachlorobenzoic acid and paraoxybenzoic aicd. The cramp threshold for cocain was determined for 3 tribes of rats (white) each of about 25 members weighing from 100-150 gm, by means of iv. injections in increasing amounts of 1% cocaine solution. The injection rate approximately was 0.1 cc/100gm-30 seconds. Cocaine injections were 48 hours apart. Prior to each, the animals were not fed for 12 hours. The percentage of animals subject to cramps was graphically plotted for each tribe as a function the cocaine dose and the accuracy of these "fundamental cocaine curves" was tested against the determination of unknown cocaine concentrations. The substances to be tested for anticonvulsive effects were ingested 30 seconds prior to cocaine injection if administered iv. and 45-180 minutes before if by pharynx bougie**. Thereafter we increased the cocaine dose until cramps occurred. Each fundamental curve was rechecked prior to testing of each substance; rats with necrotic tails were eliminated and replaced by animals with the same cramp dosis.

** Orally administered substances to be tested were dissolved in aqua dest. Doses with insufficient water solubility were ingested as deposits in water by means of the pharynx bougie.

The concentrations required for iv. administration were satisfied by the given water solubility; merely when dealing with parachlorobenzoic acid was there a requirement for several drops of

For two other tribes we used an adrenaline solution of 1/7-1/8x 10^{-4} added to the cocain in order to induce cramps. This solution too was injected 0.1cc/100 gm in 30 seconds; however we did not increase the amount of liquid administered to determine the cramp dosis, rather we increased the cocain concentration, which was 0.5-0.9%. It was found necessary to keep the adrenaline dosis constant, since for simultaneous increase in cocaine and adrenaline doses, since a high percentage of the animals reached ad exitum following sudden and severe cramps and/or edema of the lungs.

Brain blood supply is measured via the arteria vertebralis in cats weighing from 2.2-3.7 kg under urethane chlorelose anesthesia (250 or 60 mg/kg)-- see Taeschler etal. However we did not prepare the vessel ahead of its entry into the neck vertebra, but only shortly before its orifice into the skull-inside, between the first and second neck vertebra. This eliminates falsifications in blood supply values because of anastomoses with other vessel areas to a large extent. Dye injections into the arteria vertebralis used for recording showed that only a small lateral branchlet which could be neglected was pulling to the neck musculature. In order to freely expose the vertebral artery, skin and musculature between the first and fourth transverse appendices were bilaterally cut; the first neck nerve was used as the reference point for finding the artery, this nerve crossing the vessel shortly before entering the vertebral canal. After carefully freeing the arteries from the connective tissue sheath commonly enveloping vertebral artery and vein, where the latter had to be protected, the right one was ligated and the left one was supplied from a carotis by means of a polyethylene tube. A bubble flow meter had been inserted for measuring the blood supply between carotis and vertebralis. The second carotis was not ligated, in order to afford ample blood supply to the brain. Blood pressure was constantly monitored from the right a. femoralis by means of a Hg manometer. Thrombocide (WZ) was used to prevent clotting. See Bubnoff etal for the solution and concentrations of the substances used. Injections were performed by means of the polyethylene tube leading to the a. vertebralis (0.1-0.3 cc in 50 seconds). In control tests, physiological cooking salt solution and the Tween 80 doses remained without effect on brain blood supply*.

First we checked the effects of benzoic acid, paraoxybenzoic acid and of the methyl, ethyl esters of paraoxybenzoic acid of parachlorobenzoic acid and microbin (WZ) -- the latter a combination of 45% parachlorobenzoic acid sodium and 55% orthochlorobenzoic acid sodium--, on the rats' cocaine-cramps.

Three tribes of white rats of 25 each were used; the mean cramp doses CD_{50} of the three tribes were 12.0, 12.2 and 12.6 mg

---

NaOH addition -- up to pH 8-9. The substances were dissolved in physiological cooking salt solution; 0.5 - 1.2 cc/100 gm of rat were injected.

*We are grateful the firm of BENEKO in Munich for their kindly making available to us thrombocide. Similarly we thank Atlas Goldschmidt of Essen for Tween 80. Bayer Ag, Leverkusen, made available sodium benzoate, microbine, parachlorobenzoic acid and the ethyl and methyl esters of paraoxybenzoic acid. We also thank Nina, Berlin, for paraoxybenzoic acid and the propyl, butyl and benzyl esters of paraoxybenzoic acid.

cocaine-kg and therefore the results lent themselves readily to comparison. Each experiment was carried out on 15-18 animals from the same tribe; the remainder served as reserve. First that dose of benzoic acid was determined, which effected a clear increase in the cocaine cramp dosage for oral ingestion. Then we determined the isoeffective doses for the other substances. A comparison was made between the effectivenss of oral and iv. administration for each preparation and for oral ingestion, the time of maximum effect was recorded in every instance (table 1).

Table 1: Time of maximum anti-convulsive effect for oral administration of benzoic acid and its derivatives.

Substance	dosis, mg/kg	maximum effect
benzoic acid	50	45 minutes
"	100	"
parachlorobenzoic acid	50	"
paraoxybenzoic acid	50	"
paraoxybenzoic acid methyl ester	20	"
"	100	180
paraoxybenzoic acid ethyl ester	20	45
"	100	180
microbine	50	30
"	100	90

For oral administration of benzoic acid, 50 mg/kg as a 6% solution introduced with pharynx bougie showed a clear anticonvulsive effect . 45 minutes after benzoic acid ingestion this effect was maximum: CD_{50} at that time had risen from 12.0 to 14.5 mg/kg. For iv. injection of the same dosis the mean cramp dosis was 14.9 mg/kg. Paraoxybenzoic acid had the same effectiveness as benzoic acid: 50 mg/kg for oral ingestion increased CD_{50} from 12.0 to 14.2 (fig. 1).

The subsequently tested ethyl ester of paraoxybenzoic acid showed a stronger cramp-weakening effect than benzoic acid: 20 mg/kg as 0.18% solution sufficed for oral ingestion to achieve the same anticonvulsive effects; CD_{50} was raised from 12.0 to 14.6 mg/kg; see fig. 1. Here again the maximum effect occurred after 45 minutes. For iv. ingestion, already 5mg/kg of ethyl ester raised the mean cramp-dosis from 12.0 to 14.7 mg/kg (fig. 1).

Thus the relationship between the effectiveness of benzoic acid and paraoxybenzoic acid ethyl ester was not the same, neither for oral nor for iv. ingestion. The isoeffective doses of benzoic acid for oral ingestion were 2.5 times higher than for ethyl ester, the relation between the effectivenesses for iv. was 1:10. These differences are due to the fact that the ethyl ester is 4 times more effective for iv. injection, in contrast to benzoic acid.

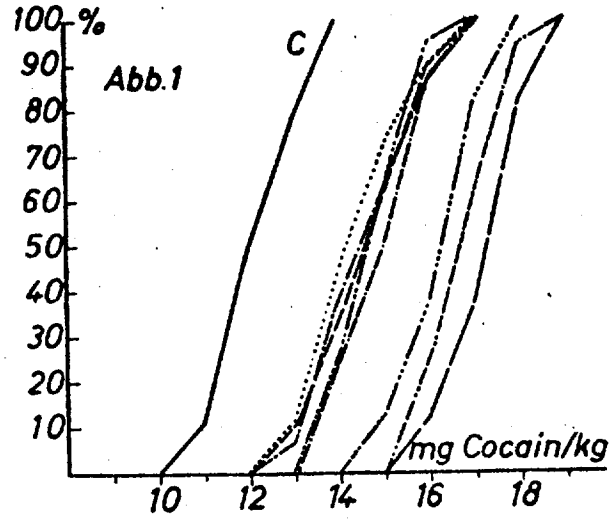
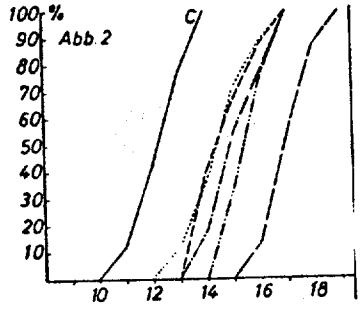


Fig. 1. Anti-convulsive effect of 50 mg paraoxybenzoic acid/kg po. (...); 20 mg paraoxybenzoic acid ethyl ester / kg p. (---); 50 mg of benzoic acid /kg po. (-.-.); 5 mg paraoxybenzoic acid ethyl ester/kg iv. (-..-..); 50 mg benzoic acid/kg, iv. (--.--.); 100 mg benzoic acid / kg po. (-'''-); 100 mg paraoxybenzoic acid ethyl ester / kg po. (--..--) and 20 mg paraoxybenzoic acid ethyl ester iv. (— —) for rat's cocaine cramp. C = cocaine basic curve; abscissa: cocaine dose in mg/kg; ordinate: percentage of animals subject to cramps. See text for further details.

For higher oral doses, the anticonvulsive efficiencies of ethyl ester and benzoic acid were comparable. 100 mg of ethyl ester/kg ingested as deposit in water with the pharynx bougie did have an appreciably stronger effect than 20 mg/kg; 3 hours after administration the CD_{50} had risen from 12.0 to 16.7 mg/kg. However the same benzoic acid dosis caused an increase of 4.3 mg/kg in the average cramp dosis and therefore was only slightly less effective; see fig. 1 "hen increasing the iv. injected amount of ethyl ester, the anticonvulsive effect relatively increased more: the iv. injection of 20 mg of ethyl ester /kg led to an increase in CD_{50} from 12.0 to 17.3 mg/kg and thus was more effective than the five-fold dosis (see fig. 1).

Paraoxybenzoic acid methyl ester and parachlorobenzoic acid were tested in the second tribe with a CD_{50}=12.2 mg/kg. Again methyl ester was administered orally and iv. Here again 20 mg/kg per os had the same effect as 5 mg/kg iv, as was already the case for ethyl ester: in both cases the average cramp-dosis rose from 12.2 to 14.4 mg/kg. 100 mg /kg of methyl ester per os corresponded in their effect to about the same dose of benzoic acid or ethyl ester (rise of CD_{50} to 16.9 mg/kg; see fig. 1 and 2). 50 mg/kg of parachloro benzoic acid per os were about as effective as 50 mg/kg of benzoic acid. Iv. injection of the same dose led only to a slight reinforcing effect with respect to anti-convulsion (fig. 2), as contrasted with methyl and ethyl esters. For oral ingestion CD_{50} was 14.9, and for iv. administration it was 15.5 mg/kg. The strongest effects occurred for oral ingestion of 20 gm of methyl ester and of 50 mg of paraoxybenzoic acid /kg 45 minutes after administration, and for 100 mg of methyl ester/kg 3 hours thereafter.



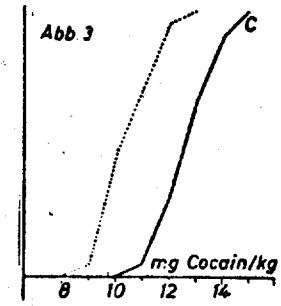


Fig. 2: Anticonvulsive effect of 20 mg/kg po. of paraoxybenzoic acid methyl ester (...); 5 mg/kg iv. of paraoxybenzoic acid methyl ester (———); 50 mg/kg of po. of parachlorobenzoic acid (-.-.); 50 mg/kg iv. of parachlorobenzoic acid (-··-··); and 100 mg/kg of paraoxybenzoic acid methyl ester, po. ((—— ——); rats' cocaine cramps. C= cocaine reference curve; abscissa: cocaine dosis in mg/kg; ordinate: percentage of animals subjected to cramps. See text for details.

Fig. 3: Effect of 100 mg/kg of po. microbine (...) on rats' cocaine cramp. C= cocaine; abscissa: cocaine dosis in mg/kg; ordinate: percentage of animals subjected to cramps. Note the cramp-reinforcing effect of microbine.

We examined the effect of microbine in the third tribe with respect to the rats'cocaine-cramps. We observed to our surprise there was an increase in the cocaine effect beyond 50 mg/kg per os. This effect became clearer yet after oral administration of 100 mg.kg: the mean cramp dosis after 1-1½ hours was reduced from 12.6 to 10.3 mg/kg (fig.3).

In further tests we made use of a combination of cocaine and adrenaline for inducing cramps.

In such cases, ½-1/3 of the usual cocain amounts sufficed to induce cramps; the differences in cocain amounts as required by the individual animals were very slight so that the cramp curves became very steep and so that the curves obtained from testing no longer intersected the reference curve (see fig. 4,5). In order to obtain the cramp-dose, we increased the amount of cocaine from test to test by only ½mg/kg, that is, each time the cocaine concentration was increased only by ½%. The average cramp dose of the first tribe was 5.1, that of the second 6.1 mg/kg.

First 20 mg/kg of methyl ester, 20 mg/kg of ethyl ester and 50 mg/kg of benzoic acid were tested and compared for oral ingestion. The CD_{50} increased from 5.1 to 7.1 mg/lg (methyl ester), to 7.0 mg/kg for ethyl ester or 6.95 mg/kg for benzoic acid. Therefore the ratio of effectiveness was 2½:2½:1, that is, the same as with cocaine alone. Since already 5.5 mg/kg of cocaine without any pre-treatment led to cramps in all animals, but since 6.0 mg/kg of cocaine after treatment with benzoic acid, methyl and ethyl esters of paraoxybenzoic acid did not induce cramps, such results were particularly impressive (fig.4,5)

Finally 20 and 100 mg/kg of ethyl ester orally administered were tested and compared in the second tribe. The higher dose led to an

increase in the anti-convulsive effect as had been the case for cocaine used alone; however in this instance the effect was not as pronounced; see fig. 1 and 6.

The effects of benzoic, parachlorbenzoic and paraoxybenzoic acids and of methyl, ethyl, propyl, butyl, and benzyl esters of paraoxybenzoic acid on the brain's blood supply was tested in 30 cats (fig. 6,7). Upon injection into the a. vertebralis, all substances led to an improvement of brain blood supply. The magnitude of the vascular enlargement achieved was a function of of the dose and of the tonus of the brain vessels; upon increasing the vascular tonus by super-ventilation, particularly pronounced vascular spasmolytic effects could be observed. We examined in every animal from 2 to 4 substances that had been injected in alternating sequence into the a. vertebralis in order to determine their efficiencies. Repeated injections in control tests for unchanged blood pressure and same initial blood supply always produced the same vascular enlargement -- see table 2.

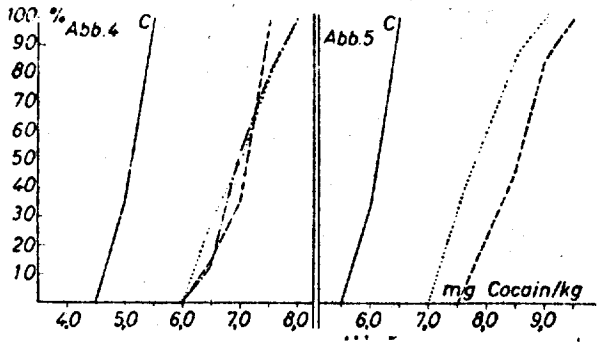


Fig. 4: Effects of 20 mg/kg of paraoxybenzoic acid ethyl ester pO. (...); 20 mg/kg of paraoxybenzoic acid methyl ester po. (---) and of 50 mg/kg of benzoic acid po. (-.-.-) on adrenaline reiforced cocaine cramps. C= cocaine reference curve; abscissa: cocaine dose in mg/kg; ordinate: percentage of animals subjected to cramps. See text for further details.

Fig. 5: Effects of 20 mg/kg (...) and 100 mg/kg (---) of po. paraoxy-benzoic acid ethyl ester on adrenaline-reinforced cocaine cramps. C= cocaine reference curve; qbscissa: cocaine dosis in mg/kg; ordinate: percentage of animals seized by cramps. See text for further details.

The iv. injections led to the same difficulties in evaluating vascular width as encountered in the simultaneously executed purine body tests (cf Taugner, Bubnoff, Weth, Waltz, Hochrein, Bartels & Schmid): low doses with little or insufficient effects on blood pressure either left the brain blood supply unaffected or only improved it slightly. But higher doses led to appreciable lowerings in blood pressure which might have left the blood supply unchanged or possibly

slightly, pressure-passively decreased*. After administrating benzoic acid and microbine (WZ) there were occasional increases in blood pressure which made evaluation of vessel musculature condition also difficult (cf Bubnoff, Schnell & Schmid). However neither iv. nor ia. (intra-arterial) administration of benzoic acid and of its derivatives led to a decrease in brain blood supply that was independent of blood pressure.

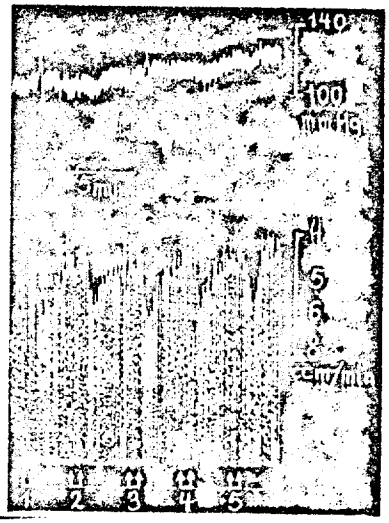


Abb. 6: Wirkung von Benzoesäure, Paraoxybenzoesäure und Parachlorbenzoesäure auf die Durchblutung des Gehirns d. Katze. Oben: Blutdruck; unten: Gehirndurchblutung (Abfall der Kurve bedeutet Mehrdurchblutung). 1, 3, 5 = 7 mg Natriumbenzoat/kg; 2 = 7 mg paraoxybenzoesaures Natrium/kg. 4 = 3,5 mg parachlorbenzoesaures Natrium/kg. Alle Injektionen erfolgten in die A. vertebralis.

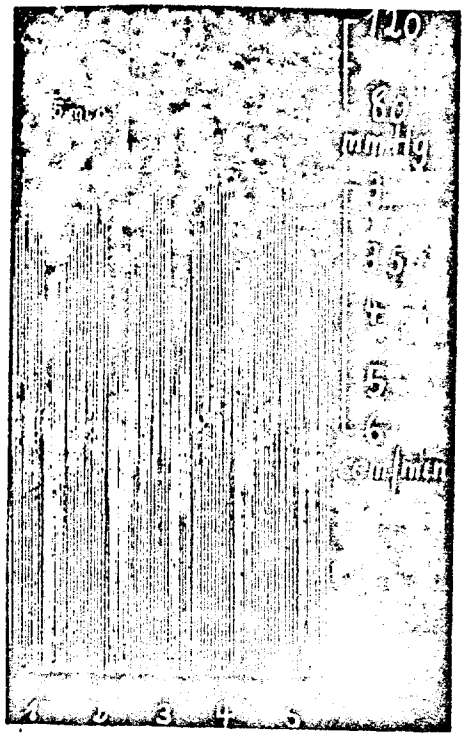


Abb. 7: Wirkung von Paraoxybenzoesäure-Methyl-, Aethyl- und Propyl-Ester auf die Durchblutung des Gehirns der Katze. Oben: Blutdruck; unten: Gehirndurchblutung (Abfall der Kurve bedeutet Mehrdurchblutung). 1, 3, 5 = 0,3 mg Paraoxybenzoesäure-Aethylester/kg; 2 = 0,3 mg Paraoxybenzoesäure-Methylester/kg; 4 = 0,3 mg Paraoxybenzoesäure-Propylester/kg. Alle Injektionen erfolgten in die A. vertebralis.

Fig. 6: Effects of benzoic, paraoxybenzoic and parachlorobenzoic acids on cats' brains. Top: blodd pressure; bottom: brain blood supply (decrease in curve indicates larger supply). 1,3,5 = 7mg/kg of sodium benzoate; 2= 7 mg/kg of paraoxybenzoic acid sodium; 4= 3.5 mg/kg of parachlorbenzoic acid sodium. All injection into the a. vertebralis.

Fig. 7: Effects of methyl, ethyl and propyl esters of paraoxybenzoic acid on the cat's blood supply. Top: blood pressure; bottom: brain blood supply (drop in curve indicates increase in supply). 1,3,5 = 0.3 mg/kg of paraoxybenzoic acid ethy ester; 2= 0.3 mg/kg of paraoxybenzoic acid methyl ester; 4= 0.3 mg/kg paraoxybenzoic acid propyl ester. All injections into the a. vertebralis.

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*According to the results of Matthews etal, the lowering of the blood-pressure following iv. ester injection seem to be due among other causes to the effect on the cardiac muscle ("myocard depression").

sodium benzoate	17, 7, 9, 16, 8, 8, 9, 16, 6, 9, 16, 12, 16, 7, 6, 6, 16, 7, 8
p-chlorobenzoic acid sodium	12, 3,5, 4
p-oxybenzoic acid sodium	8,5, 8,5, 16, 7, 6,5, 6,5
methyl ester of paraoxybenzoic acid	0,7, 0,3, 0,4, 0,3, 0,6, 0,3, 0,4, 0,3, 0,8, 0,8
ethyl ester of paraoxybenzoic acid	0,3, 0,4, 0,4, 0,7, 0,8, 0,4, 0,3, 0,3, 0,3, 0,6, 0,3, 0,4, 0,4, 0,4, 0,3, 0,8, 0,3
propyl ester of paraoxybenzoic acid	0,3, 0,3, 0,4, 0,8
butyl ester of paraoxybenzoic acid	0,7, 1, 0,5, 0,3, 0,7, 0,3
benzyl ester of paraoxybenzoic acid	0,6, 0,5, 0,5
salicylic acid sodium	8,5, 14
acetate salicylic acid Na *	8,5, 17
ratio	1:1, 1:1, 1:2, 1:1,25, 1:1,25, 1:1, 1:1,25, 1:1,25, 1:1, 1:1, 1:1, 1:1, 1:1, 1:1, 1:1, 1:1, 1:1, 1:1, 1:2, 30:1, 20:1, 20:1, 20:1, 30:1, 20:1, 20:1, 30:1, 20:1, 15:1, 1:1, 1:1, 1:1,25, 1:1,25, 1,5:1, 2:1, 2:1

*) Die beim Aspirin (WZ) angegebenen Dosen sind auf den Salicylat-Gehalt umgerechnet.

Table 2. Tests on cats' brain vessels. Doses causing equal vessel expansion, sodium benzoate, parachlorobenzoic acid sodium, paraoxybenzoic acid sodium, methyl, ethyl, propyl and butyl esters of paraoxybenzoic aicd, sodium salicylate and acetyl salicylic acid sodium (aspirine-Na, WZ), in mg/kg of injection into a. vertebralis.

For a dose of 5 mg.kg ia., zodium benzoate showed clear vascular spasmolytic properties; amounts of 6-17 mg/kg led to strong vasodilatations that were convenient for comparison tests. The effectiveness of paraoxybenzoic acid was about the same as benzoic acid's: for 8-16 mg/kg the same effects could be achieved as by means of the above described injections of benzoate doses. M.crobine's (WZ) vascular spasmolytic effects were not tested for in the cat's brain (see further below). Though parachlorobenzoic acid (4-12 mg/kg) also was somewhat stronger in effectiveness in the cat's brain vessels than benzoic acid (ratio of sodium benzoate to parachlorbenzoic acid sodium = 1:1½-2), such differences were not as clear as they had been in the kidney vessels (see Bubnoff etal). Methyl, ethyl, propyl, butyl and benzyl esters of paraoxybenzoic acid were used in doses of 0.5-0.6 mg/kg. The effectiveness of the esters was comparison-tested in 15 experiments and found to be nearly the same; only for benzyl ester did the isoeffective doses lie at a level 1½-2 as high as for the other esters. Again all esters were appreciably more effective than sodium benzoate: the ratio of efficiencies in ten tests on the average was 22½:1 (15-30:1). Sodium salicylate and aspirin (8-10 mg/kg) too showed vessel-expanding properties; the effectiveness of these substances corresponded approximately to that of paraoxybenzoic acid.

In our above described tests in the vessels of kidneys and extremities, no changes in blood pressure could be detected for ia. injections in cats. However there were frequent drops following injections into the a. vertebralis, more rarely there were increases. These changes which probably were centrally determined in general amounted no more than 5-10 mm. For blood pressure effects beyond this scope, there was no evaluation for the pertinent injections with respect to comparison tests. On two occasions, microbine (WZ) caused a rise in blood pressure for each injection in the amount of 20-35 mm

and therefore could not be tested for its vessel-expanding properties in the brain vessels.

Notes during proof-reading: Benz in his orientation tests examined the esters of paraoxybenzoic acid for their local-anaesthetic effects. For that purpose aqueous solutions of these esters were placed into the oral cavities of 20 members of the Heidelberg Pharmacological Institute. For positive reactions there followed within a few minutes a numb or furry feeling at the tongue. The following results were obtained:

	methyl ester	ethyl ester	propyl ester	butyl ester
solution concentration	0.1%	0.05%	0.03%	0.02%
positive reaction	17	18	16	5
dubious reaction	1	1	3	13
no effect	2	1	1	2

The individual variability in reaction may be due to the low concentration of the ester solutions used.

DISCUSSION:

According to these results, parachlorobenzoic and paraoxybenzoic acids and the methyl and ethyl esters of the latter, besides benzoic acid, show clear anticonvulsive properties for oral administration. The isoeffective doses of benzoic, parachlorobenzoic, paraoxybenzoic acids and of the methyl and ethyl esters of paraoxybenzoic acid are in ratio of approximately 2½:2½:2½:1:1. The effective benzoic acid dose corresponds (50 mg/kg) to that found by Zwissler. For iv. administration, the differences in efficiencies were even more pronounced, the ratio of benzoic acid to ester being about 1:10; benzoic acid and parachlorobenzoic acid for both kinds of applications were about equally effective. But methyl and ethyl esters were roughly 4 times more effective for iv. administration. Metzger etal, also Schuebel & Manger, found no paraoxybenzoic acid esters in the droppings following feeding, nor any free acids. The research from other authors too indicates that there is complete reabsorbtion of these substances from the intestine (Sokol, Jones etal; also see Sabalitschka etal). However the paraoxybenzoic acids are very rapidly hydrolyzed in the body* (Sokol, Richardson etal): for an iv. injection of 50 mg/kg of esters, these were already present in the plasma and only in the form of free or combined paraoxybenzoic acid after 5-30 minutes. For oral administration of 1 gm/kg too, the concentrations of the esters in the plasma when compared with free paraoxybenzoic acid, was only minor. The lesser oral effectiveness of methyl and ethyl esters in our experiments is likely to be mostly due to the rapid hydrolysis, the largest part immediately after iv. administration being still unsaponified. But since the two esters were still 2½ times as effective for oral ingestion as benzoic and paraoxybenzoic acids, it seems that a part of the ester responsible for the higher effectiveness remains unsaponified in the plasma.

For oral ingestion of ethyl esters or benzoic acid in doses as high as 100 mg/kg, we no longer could clearly distinguish differences in effectiveness. For such doses the time from taking effect till reaching the maximum anti-convulsive effect was about 3 hours. Appa-

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*According to in vitro experiments by Richardson etal, hydrolytic fission of the esters mainly occurs in the liver and kidney.

rently during this very long reabsorption time for such ester quantities, a fairly large part is saponified as compared to the part when there is more rapid reabsorption of smaller doses. This would explain how the differences in efficiencies for ethyl ester with respect to iv. and oral ingestion clearly increased with dosing. Microbine (WZ) was the only benzoic acid derivative that surprisingly caused reinforcement of cocaine cramps. This might be relate to the increase in blood pressure observed in cats for iv. and intravertebrate administration; blood-pressure increasing substances may also increase the cramp-effectiveness of cocaine (Keil & Ruehling).

All the benzoic acid derivatives tested by us in the cats' brain vessels clearly were endowed with vascular-widening properties. For reasons advanced earlier, we again preferred the ia. injection. The vessel-widening efficiency of benzoic, paraoxybenzoic and parchlorobenzoic acids and of the methyl, ethyl, propyl, butyl and benzyl esters of paraoxybenzoic acid were in the ratios of 1:1.2:1.7:22.5:22.5:22.5:22.5:15**. The esters' effective doses (0.3 -0.7 mg/kg) approximately corresponded to the simultaneously executed tests with purine for a theophylline derivative(KGG 158) substituted into an 8 position and containing an amino group and for theophyyline ethylene diamine (Euphyylin, WZ), that is, for the values thus obtained, (0.18-0.9 and 0.25-1.5 mg/kg resp.; Taugner etal). As was the case for the purine substances, only slight effects could be achieved for iv. injections. In no test were active vasoconstrictions observed.

Table 3 shows the efficiencies of the tested benzoic acid derivatives for the kidney, extremity and brain vessels of cats, the "lissive" efficiencies for the chewing musculature of rats, and the anti-convulsive efficiencies for iv. and po. administrations.

	benzoic acid	paraoxy-benzoic acid	paraoxy-benzoic acid ester**	para-chloro-benzoic acid	micro-bine*
kidney blood supply	1	1-2	30-100	3-8	½-2
extremities blood supply	1	***	20-30	***	1
brain blood supply	1	0.8-1	15-30	1½-2	***
anticonvulsive effect					
oral ingestion	1	1	2½	1	☺
iv. ingestion	1	***	10	1	☺
"lissive" effect	1	1-1½	10=100	2-4	1-1.7

Table 3: efficiencies of individual substances compared to benzoic acid.

** : methyl, ethyl, propyl, butyl, and benzyl esters of paraoxybenzoic acid. Benzyl ester was slightly less effective than the other ester. The effect on blood supply of extremities was tested only for the methyl, ethyl and propyl esters, and the anti-convulsive effect only for the methyl and ethyl esters.

*** : not determined

☺ : In contrast to the other substances, Microbine for peroral ingestion led to reinforcement of the cocaine cramps.

** When converting the substances injected as sodium salts into the content of free acid, one obtained approximately the same relation. A conversion to equimolar doses too leads to no appreciable change of the above indicated ratio of efficiencies.

As seen from table 3, the paraoxybenzoic acid esters were relatively the most effective in the kidney vessels; the ratio of benzoic acid to ester in the brain and extremity vessels was approximately equal (1:15 -30 and 1:20-30 resp.). The esters' anti-convulsive effectiveness was 10 times stronger for iv. injection than it was for benzoic acid; this ratio too was of the same order of magnitude. The effectiveness ratio obtained in the brain vessels for theophylline, coffeine and theobromine approximately corresponded to the ratio found by Eichholtz etal for the rat cocaine-cramp, (1:0.9:0.7 and 2:2:1 resp.) Eichholtz etal attributed the anticonvulsive effect of these substances to the widening of the vessels at the central nervous system. Possibly there is a relation between vessel widening and anticonvulsive effect in benzoic acid and its derivatives too.

The preparations of the benzoic acid series also show vascular spasmolytic, "lissive", ganglioplegic and anticonvulsive effects. The latter could also be demonstrated for oral administration. For all test procedures, the paraoxybenzoic acid esters were appreciably more effective than benzoic acid itself. As already reported in the first communication, the usefulness of these compounds as preservatives should be discussed in future communications and they should take into account these conditions since it was shown that demonstrable effects arise also for oral ingestion, or may arise. Again the feasibility of therapeutic applications is stressed herewith.

SUMMARY

Continuing previous research, the effects from benzoic, parachlorobenzoic and paraoxybenzoic acids and of their esters on the cocaine-cramp of rats and on the brain blood supply of cats were tested.

Benzoic, parachlorbenzoic and paraoxybenzoic acids and the methyl and ethyl esters of paraoxybenzoic acid showed clear anticonvulsive properties for iv. and oral administration. Both esters were 10 times more effective for iv. ingestion than benzoic acid. the ratios of the anti-convulsive efficiencies of benzoic, parachlorobenzoic and paraoxybenzoic acids and of the methyl and ethyl esters of paraoxybenzoic acid for oral administration are 1:1:1:2½:2½. The iv. effective dose of the esters was 5 mg/kg, and that orally effective was 20 mg/kg.

Besides the previously observed vascular spasmolytic effects in kidney and at the extremities, such were also observed this time in the brain vessels. The efficiencies determined with respect to the vascular spasmolytic of benzoic, paraoxybenzoic and parachlorobenzoic acids and the methyl,ethyl, propyl, butyl and benzyl esters of paraoxybenzoic acid for a. vertebralis injections were in the ratios of 1:1.2:1.7: 22.5:22.5:22.5:22.5:15.

Again we thank Prof Eichholtz for his multi-faceted encouragements and many suggestions.

Summary:

Studies on the Pharmacology of Benzoic Acid, Para Chlorbenzoic Acid, Parahydroxybenzoic Acid, and its Esters

In continuance of former investigations the action of benzoic acid, parachlorbenzoic acid, and parahydroxybenzoic acid, and its esters on cocaine convulsions in rats and on cerebral blood flow in cats have been investigated. Benzoic acid, parachlorbenzoic acid, parahydroxybenzoic acid, parahydroxybenzoic acid methyl- and ethyl esters showed marked anticonvulsive action following intravenous and oral application. Following intravenous injection, the two esters were about 10 times more effective than benzoic acid; following oral application the relation of anticonvulsive action in benzoic acid, parachlorbenzoic acid, parahydroxybenzoic acid, parahydroxybenzoic acid methyl and -ethyl ester was 1:1:1:2,5:2,5. In the esters the effective intravenous dose was 5 mgs, the oral effective dose was 20 mgs/kg body weight. In addition to the vascular spasmolytic action formerly observed in the kidneys and extremeties, spasmolytic action was also observed in cerebral vessels. The proportion of spasmolytic action following injection into the a. vertebralis, in benzoic acid, parachlorbenzoic acid, parahydroxybenzoic acid, parahydroxybenzoic acid methyl-, ethyl-, propyl-, butyl-, and benzyl esters was found to be 1:1,2:1,7:22,5:22,5:22,5:15.

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Sorbate Salts

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Sodium benzoate and sorbate salts are common food preservatives frequently found in the same product. Their determination, in the acid form, generally requires complete separation from each other. There are several methods available for the determination of these acids (1), the precision or speed of which may not be sufficient for all purposes.

Recent studies by the author, involving an elixir, have led to a method of good accuracy and precision for the determination of sodium benzoate in the presence of sorbates. The method is based on the isolation of the two species, in their acid form, by benzene extraction and subsequent hydrogenation of sorbic acid. The resulting mixture is then passed through a partition column and eluted with solutions of acetic acid in chloroform. The column work is a modification of that described by Weber (2). A standard benzoic acid solution is carried through the hydrogenation and column steps. Quantitative results are obtained by UV spectrophotometry.

Experimental

Apparatus and Reagents

(Use reagent grade solvents throughout the method.)

(a) *Hydrogenation system.*—Pass hydrogen gas through Nesbitt absorption bulb containing benzene, then into two 50 ml centrifuge tubes containing the samples and connected in series. *See* reference (3) for additional details.

(b) *Chromatographic column.*—20 mm id. with fritted glass disk.

(c) *Acid-washed diatomaceous earth.*—Celite 545 (Johns-Manville).

(d) *Palladium catalyst.*—5% on $CaCO_3$ (Engelhard Industries, Newark, N.J.).

(e) *Benzoic acid standard solution.*—0.03 mg benzoic acid/ml benzene.

(f) *Eluting solvents.*—Water-saturated benzene, chloroform, and ether.

Column Preparation

Prepare diatomaceous earth:water mixture (1:0.75) by triturating diatomaceous earth with water. Prepare diatomaceous earth:bicarbonate mixture by triturating 4 g diatomaceous earth with 2 ml freshly prepared 1N $NaHCO_3$. Add 0.5 g diatomaceous earth:water mixture to bottom of column, tamping down firmly. Triturate 6 g diatomaceous earth:bicarbonate mixture with benzene and transfer resulting slurry, in three approximately equal increments, to column, tamping down firmly with each addition.

Procedure

Extract 3 ml sodium benzoate solution containing 1 mg/ml by shaking 2 min with 1 ml 5N HCl, 6 ml water, 25 ml benzene, and 3 g finely ground anhydrous Na_2SO_4. Let phases separate and pass benzene phase through 20 g anhydrous Na_2SO_4 into 100 ml volumetric flask. Repeat extraction 3 times. Divide 50 ml of sample in two approximately equal parts into two 50 ml centrifuge tubes; add 0.025 g Pd catalyst to each tube. Hydrogenate samples 3 min. (Initiation of hydrogenation is indicated by blackening of catalyst.) After hydrogenation, percolate 40.0 ml of well-mixed contents of the tubes through column, add glass wool plug to column, and wash column with 80 ml ether. Add 10 ml 10% glacial acetic acid in $CHCl_3$ to column, discarding first 5 ml of this eluate. Collect eluate in 50 ml volumetric flask containing 7 ml methanol and 4 drops of concentrated HCl. Complete elution by adding 1% glacial acetic acid in $CHCl_3$ to column until flask is filled to volume.

Take 50 ml standard through hydrogenation and column steps. Prepare blank by adding, to 50 ml volumetric flask, 7 ml methanol, 4 drops concentrated HCl, and enough 1% glacial acetic acid in $CHCl_3$ to fill to volume. Using 2.5 cm cells, measure spectra of samples and standard on recording spectrophotometer from 340 to 250 mμ vs. blank. Construct baseline, using tangent method, from absorbance minimum at ca 255 mμ tangent to curve at ca 287 mμ. (Absorbance maximum is at ca 273 mμ.) Calculate mg sodium benzoate/ml by comparison of net sample and standard absorbances, using the factor 1.18 to convert the acid value to that of the salt.

Results and Discussion

Sodium benzoate recoveries from samples containing 1 mg sodium benzoate/ml and 2 mg potassium sorbate/ml in an aqueous and an elixir mixture were 99 ± 0.85% (6 determinations) and 100 ± 1.95% (5 determinations), respectively. The combined recovery was 99.5 ±

1.45%. The data for both mixtures were subjected to an F test. Since no significant difference in the standard deviations was found, the recovery and standard deviation were reported from the combined data.

At one point in the work described a study was made of the effect of the amount of catalyst on recovery. A definite correlation was found. By adding 40.0 ml of the *well-mixed* contents of the tube, four-fifths of the catalyst is added to the column. Since the same procedure is followed with the standard, there is no source of error.

The determination of benzoic acid in the presence of sorbic acid, described herein, is based on the elimination of the $\pi \rightarrow \pi^*$ transitions of the conjugated diene, sorbic acid. Under the conditions described, the benzenoid absorption of benzoic acid is unaffected.

The amount of salting out required for complete extraction of benzoic acid will depend on the nature of the sample. In this work, the water mixture required virtual saturation of the aqueous phase with sodium sulfate, whereas the elixir did not require sodium sulfate.

Under the present conditions, the ether wash physically transports some sodium bicarbonate down the column. The product of the acid-basic salt reaction is sodium acetate which, presumably, in amounts below its solubility limits in chloroform, results in baseline difficulties. Satisfactory measurements were obtained by a column having a diatomaceous earth:water packing at the bottom of the column and the tangent method for obtaining a baseline. The latter is valid as the interference has been found to be linear with wavelength in the region scanned.

The accuracy and precision of this method should be satisfactory for most cases.

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POLAROGRAPHIC DETERMINATION OF BENZOIC ACID

Benzoic acid and its salts belong to the preservatives used most frequently in the food industry. Despite their extensive and extended use, their analytical determination has not yet been satisfactorily solved. The previously used methods of determination are not satisfactorily specific in most cases and their execution is not always appropriate. The anlytical means are complicated and require much time. In each method it is necessary to isolate benzoic acid by extraction from an organic solution.

Gravi-, titri-, and colorimetric methods can be used for benzoic acid determination, of which the colorimetric are relatively the most specific. The method described by Spanyar et al. is very interesting: It describes the reaction of the benzoic acid nitro-derivative with ethanol and acetone. The coloration produced during the reaction is stable for 90 min at 0 degrees C. and at lower concentrations it follows the Beer-Lambert law. According to the authors the reaction is specific and is not disturbed by other substances in preserved foods.

The largest errors in the analysis of benzoic acid occur during extraction of the tested material. Spanyar considered the problem extensively and worked out special extraction procedures for many food types, rich in sugar, fat, and protein among other things.

Since it is known that nitro bodies are suitable for polarographic determination, we used the nitration of benzoic acid for it, in order

to make possible its polarographic determination. The benzoic acid-nitroderivative is reduced in acidic solutions; this reaction shows up well on the polarogram and can be used analytically. The optimal conditions for the nitration of benzoic acid and for its polarographic determination are described in this paper.

Experimental Part

Apparatus and reagents

Measurements were taken with a polarograph, Heyrovsky system, Type V 301 b, made in the Zbrojovka factory with an automatic recording-instrument EZ 2 and with a Kalousek container with a separated, saturated mercury (I) sulfate electrode. The capillary had a reservoir height of h=60 cm and a flow rate of 3.1 mg/sec as well as a drip duration of 2.1 sec.

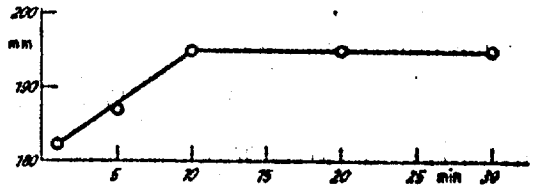


Fig. 1. Nitration of benzoic acid in dependence on time. Final concentration of benzoic acid: 5×10^{-4} mole. Final concentration of the nitration mixture: 20%. Nitration temperature: 100 deg. C. Nitration duration: 5, 10, 20, 30 min. Sensitivity 1:70, saturated mercury (I) sulfate electrode, h=60 cm.

Solutions and reagents

1. 0.005 m-benzoic acid solution in methanol
2. nitration mixture: sulfuric acid and nitric acid in a 1:1 ratio
3. 1% sodium hydroxide solution in methanol
4. 25% sulfuric acid
5. ether

All of the chemicals were used in their purest obtainable form. First of all, it was necessary to ascertain the optimal nitration conditions. One obtains this with a nitration mixture of equal parts H_2SO_4 and HNO_3. Under these conditions, the benzoic acid nitroderivative yields a good polarogram. With higher concentrations of nitric acid in the nitration mixture, another step can be observed on the polarogram, which is, however, dependent of the concentration of the nitric acid and on the ratio of benzoic to nitric acid. One ml was determined as the optimal amount of nitration mixture. This amount is necessary, both from the standpoint of polarography and with regard to ease of manipulation (Table 1). Nitration occurs quickly and is relatively independent of the temperature, though one receives fewer reproducible results at higher temperatures. For this reason, the nitration is carried out in a boiling water bath. (Table 2). Under these conditions, nitration occurs very quickly and is practically completed in 10 min. Lengthening the nitration time does not alter the step height. In our work we used a nitration time of 15 min. The resulting derivative is stable in the acid solution and does not change even after 20 hours. The calibration curve was determined under these conditions.

Table 1

Step height dependence of the amount of nitration mixture added. Weighted sample: 0.61 mg benzoic acid.

Curve No.	Nitration mixture ml	Step height mm
1	0.5	154
2	1.0	166
3	1.5	159
4	2.0	143
5	5.0	151

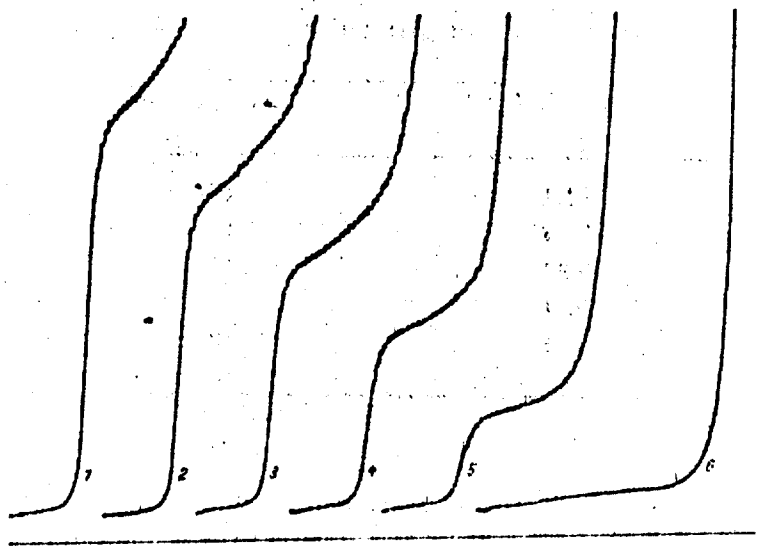
Table 2

Step height dependence of the reaction temperature during nitration. Weighted sample: 0.61 mg benzoic acid.

Curve No.	Temperature deg. C	Step height mm
1	20	127
2	40	124
3	50	115
4	70	120
5	90	130
6	100	141

Procedure

The benzoic acid standard solution is pipetted in amounts of 1.0-0. $1x10^{-3}$ mole into the test tubes, 1 ml of the 1% sodium hydroxide solution in methanol is added and evaporated in a boiling water bath. 1 ml nitration mixture is added to the evaporation residue, so that the entire evaporation residue is moistened and then nitrated on the boiling water bath within 15 min. Then the reaction mixture is cooled, 3 ml water is added, again cooled, transferred into a 10 ml volumetric flask, 0.5 ml of 0.5% gelatin solution are added and is filled up to the mark with water. Then the solution is thoroughly mixed and poured into the Kalousek polarographic container. The oxygen is expelled by a nitrogen current and then the polarographic curve is recorded (Ill. 2).



Ill. 2. Polarographic calibration curve for benzoic acid. Final concentrations of benzoic acid in the polarographed solution: (1) 5; (2) 4; (3) 3; (4) 2; (5) 1; (6) 0.0×10^{-4} mole. Final solution of the nitration mixture: 20%. Sensitivity 1:70. Mercury (I) sulfate electrode h = 60 cm, 180 m V/Absc.

This procedure was tested by analysis with standard solutions of pure benzoic acid. All results obtained were reproducible. Equally good results were reached by model analysis of preserved foods, primarily juices and syrups.

For the isolation of the samples prepared in this way the method of Spanyar was used (Compare 4, p. 123).

From the extracts prepared in this way, 1 ml was measured off with a pipette, 1 ml of 1% sodium hydroxide solution in methanol was added, and the procedure was continued as described above. The obtained measurements are recorded in Table 3.

Table 3

Polarographic determination of benzoic acid in fruit products

Product	No. of Analyses	Benzoic Acid Added mg/100g	Benzoic Acid Detected mg/100g	Difference mg	Difference %
1. Lemon juice	4	100.0	111.0	+11.0	+11.0
2. Orange juice	3	90.0	92.8	+ 2.8	+ 3.1
3. Orange juice	3	130.0	141.1	+11.1	+ 8.5
4. Orange juice	3	156.0	153.2	- 2.8	- 1.7
5. Apple cider	3	110.0	115.0	+ 5.0	+ 4.5
6. Apple cider	3	140.0	138.7	- 1.3	- 1.0
7. Apple cider	3	100.0	107.7	+ 7.7	+ 7.7
8. Cherry juice	2	117.3	124.5	+ 7.2	+ 6.0
9. Cherry juice	2	126.6	139.5	+12.8	+10.0
10. Strawberry juice	2	103.0	105.0	+ 2.0	+ 1.9
11. Raspberry syrup	2	250.0	253.5	+ 3.5	+ 1.4
12. Raspberry syrup	2	170.0	179.5	+ 9.5	+ 5.5
13. Raspberry syrup	3	110.0	123.9	+13.9	+12.6
14. Strawberry syrup	2	140.0	152.0	+12.0	+ 8.5
15. Rose hip syrup	3	135.0	129.0	- 6.0	- 4.4
16. Rose hip syrup	2	112.5	123.5	+11.0	+10.0
17. Strawberry jam	2	100.0	110.0	+10.0	+10.0
18. Celery juice	2	115.0	110.0	- 5.0	- 4.3
19. Celery juice	2	130.5	122.0	- 8.5	- 6.5
20. Tomato juice	2	145.3	130.0	-15.3	-10.3
21. Tomato juice	2	132.7	130.5	- 2.2	- 1.2

Discussion

This polarographic procedure for the determination of benzoic acid shows several advantages over the methods used up to this time: primarily, speed and simplicity. It is appreciably shorter in comparison with the colorimetric determination method because the nitration derivate in the diluted nitration mixture is polarographically reduced, whereas in the Spanyar method, the resulting nitroderivative is isolated by several extractions, evaporated and treated in an acetone-alcohol mixture. The stability of the color reaction is not fully satisfactory, although it does not vary at a temperature of 0 degrees C for 90 min, which is not advantageous from an analytical standpoint. In the polarographic determination the nitroderivative is still stable after 20 hours. The results obtained with this method are recorded in Table 3.

The standard deviation for 10 values of benzoic acid detected in apple cider was ± 3% with a distribution of ± 6% at 95% statistical reliability.

Benzoic acid can be determined with satisfactory accuracy according to these studies, which served solely to test the method. The obtained results and their reproducibility from the Spanyar method using the same samples were worse.

Difficulties can be observed in substances with a large pectin content, since benzoic acid extraction is harder to achieve. This fact, however, is not dependent on the actual polarographic determination.

Summary

A new polarographic method for benzoic acid determination after previous nitration and dilution was perfected. The optimal conditions were determined. After nitration and dilution one can polarograph directly in an acidic mixture. The formed nitroderivative is stable enough in the strongly acidic solution and remains unchanged after 24 hours. The nitroderivative of benzoic acid gives a well developed and measurable step height in the polarogram.

The method was tested both by analysis of pure benzoic acid and by analysis of foods. The polarographic determination was simpler and quicker than the Spanyar method.

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Food Research 19, 1954.

SORBIC ACID AS A FUNGISTATIC AGENT FOR FOODS. I. HARMLESSNESS OF SORBIC ACID AS A DIETARY COMPONENT[a,b,c]

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(Manuscript received June 5, 1953)

It has been discovered by Gooding of The Best Foods Laboratories *(7)* that certain of the α,β-unsaturated fatty acids are good fungistatic agents for foods and food wrappers. Thus, crotonic acid, $CH_3 \cdot CH:CH \cdot COOH$, and its homologues fall into this category. Sorbic acid $CH_3 \cdot CH:CH \cdot CH:CH \cdot COOH$, is particularly well suited as a fungistatic agent for foods. Sorbic acid may be used to prevent mold growth in food products in which sodium benzoate is now employed, *viz.* orange juice, fruit and fruit syrups, pickles, margarine, and candies. In protecting cheeses and cheese products, foods which are very susceptible to mold spoilage, sorbic acid has been found to be markedly superior to sodium benzoate as a fungistatic agent *(17)*. This has also been demonstrated in studies conducted on margarine *(7)*. Phillips and Mundt *(15)* have reported on the advantages of using sorbic acid to inhibit yeast and mold growth while permitting normal fermentation in curing cucumbers.

Over and above the finding that sorbic acid is a superior fungistatic agent, there are certain advantages which would accrue from the use of sorbic acid in place of sodium benzoate. In the first place, a smaller concentration of sorbic acid would be required to protect foods, particularly if protection of the same degree furnished by 0.1% sodium benzoate is desired. Secondly, as will be demonstrated in the second paper of this series *(2)*, the α,β-unsaturated fatty acids are readily metabolizable like any food fatty acid, while benzoic acid cannot be so utilized. Indeed, the α,β unsaturated fatty acids are intermediates in the normal oxidation of the saturated fatty acids. Sorbic acid is metabolizable in exactly the same way as is the natural saturated fatty acid, caproic acid *(2)*, both yielding carbon dioxide and water as end-products. On the other hand, benzoic acid is a strong inhibitor of fatty acid oxidation by liver enzymes *(20)* and must be detoxicated. In man, this occurs by conjugation of the benzoic acid with glycine to yield hippuric acid, $C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot COOH$. The latter compound is excreted quantitatively in the urine *(9)*. Some of the benzoic acid

[a] Carried out under a research grant from The Best Foods, Inc.

[b] Paper No. 346 from the Department of Biochemistry and Nutrition, University of Southern California.

[c] The substance of this report comprises part of the material presented as paper 82 at the Boston meeting of the Institute of Food Technologists on June 22-24, 1953.

may be detoxicated with glucuronic acid, derived from glucose, to form phenyl glucuronide *(19)*. In these ways, a compound which resists the oxidative processes of the body is coupled with normal metabolites to yield products which are then readily excreted in the urine. The liver is the site of benzoic acid detoxication, and this fact has actually led to the utilization of hippuric acid excretion as a test of liver function. Individuals with poor liver function will have difficulty in detoxicating benzoic acid.

Before sorbic acid may be used as a fungistatic agent for foods, it would have first to be demonstrated that it is harmless. Two lines of investigations were followed in evaluating this problem. The first involved carrying out conventional toxicity tests, the results of which are presented in this paper. The second involved the metabolic approach to determine the fate of sorbic acid in the animal body.

In this report are summarized the results of studies to determine the LD_{50} of sorbic acid for rats and also to ascertain whether or not this acid exerts a deleterious effect on growth of rats or dogs when fed in amounts many times greater than that required to protect foods from mold spoilage. The tissues of several groups of rats and of the dogs were subjected to histopathological examination at the termination of the test following the 3-month feeding period. An effective part of this toxicity study has dealt with a comparative evaluation of the toxicity of sorbic acid *versus* that of sodium benzoate. In this way, it was possible to assess the toxicity of a new fungistatic agent in relation to another that has been accepted for many years as a fungistatic agent for foods.

In the second report of this series *(2)*, it was possible to relate the metabolism of the unsaturated C_6 fatty acid, sorbic acid, to that of the saturated C_6 fatty acid, caproic acid; the latter is found in butter fat in concentrations of 1.5 to 2.5%. Since it has been demonstrated that the mechanism of fatty acid oxidation in the rat is exactly the same as that in man, the metabolic approach actually constitutes an evaluation of sorbic acid metabolism in the human. The demonstration that the metabolism of sorbic acid and caproic acid is identical in the animal organism is unequivocal proof of the harmlessness of sorbic acid.

The present paper presents the findings obtained in 2 independent investigations, one carried out in the laboratories of the Department of Biochemistry and Nutrition, University of Southern California (Laboratory *A*) and the other at the Mellon Institute of Industrial Research, University of Pittsburgh (Laboratory *B*). Different strains of rats were employed in the 2 laboratories, and the chemicals under test were obtained independently. The dog tests were carried out only in Laboratory *A*.

EXPERIMENTAL AND RESULTS

The experiments of Laboratory *A* were carried out on rats of the University of Southern California strain while those employed in the tests by Laboratory *B* were animals of the Sherman strain selected from rats raised in its own laboratory.

The identity of the samples with sorbic acid[4] was established by determination of

[4]The sorbic acid employed in the tests in Laboratory *A* was either obtained directly from the Carbide and Carbon Chemicals Company, New York, after June 1951 (S-93868), or from a sample supplied by The Best Foods who had purchased it from the above source (S-33568).

melting point and equivalent weight as well as by general crystalline form and solubility tests carried out in two cases in the Southern California laboratories. Spectrophotometric absorption curves obtained in the laboratory of The Best Foods, Inc. indicated that the sorbic acid was better than 99% pure on a moisture-free basis. All the sorbic acid used by Laboratory *B* was obtained from a 10-pound sample of commercial grade sorbic acid,* labeled "Drum No. 460-R." It was received on July 4, 1950. U.S.P. sodium benzoate (Merck) was used in the toxicity tests carried out in both laboratories. The purity of the caproic acid† used in the dog tests was ascertained by the determination of equivalent weight, refractive index, density, melting point, and boiling point tests.

Acute toxicity tests. In the toxicity tests carried out in Laboratory *A*, male rats, weighing 212 to 430 grams, and female rats, weighing 163 to 267 grams were used. The method of testing was similar to that employed earlier *(3)*. This involved feeding an appropriate dose of the test substance by stomach tube to animals previously fasted for 18 hours. The animals were kept in separate cages and were provided with stock diet and water. The tests were considered positive if the animals died within 5 days. This interval was chosen since by this time practically all animals which had survived were again gaining weight, and were in satisfactory nutritional condition. Toxicity studies in Laboratory *A* were made only with the sodium salts of sorbic and benzoic acid. A total of 182 rats were used for the tests with sodium sorbate, and a total of 70 rats for those with sodium benzoate by Laboratory *A*. In the case of Laboratory *B*, acute toxicities were determined of both free sorbic acid and sodium sorbate as well as of sodium benzoate. Sorbic acid was fed as a suspension which was prepared as follows: The acid was ground fine in a mortar moistened with a few drops of 1% "Tergitol" Penetrant 7, and 0.25% agar solution was added to give a suspension containing 0.20 g. sorbic acid per ml. The suspension did not separate during 15 minutes' standing; gentle shaking resuspended it after the suspension was allowed to stand over a prolonged period. Sodium sorbate and sodium benzoate were fed in 10% aqueous solution. In the series of tests carried out by Laboratory *B*, 90 to 120 g. unfasted rats were used. There were 5 males and 5 females in each group and the animals were observed for 14 days after the dosing. The data on the toxicity tests are summarized in Table 1.

TABLE 1

Average LD_{50} of sorbic acid when fed as free acid or sodium salt and of sodium benzoate in rats

Category	Laboratory *A*	Laboratory *B*
LD_{50} for sorbic acid	.....	10.50±1.96 g./kg. (9.17-12.03)
LD_{50} for sodium sorbate (calculated as sorbic acid)	4.0 g./kg.	5.94 g./kg. (5.65-6.23)
LD_{50} for sodium benzoate (calculated as benzoic acid)	2.1 g./kg. (1.7-2.5)	3.45 g./kg. (3.15-3.74)
LD_{50} (sodium sorbate) : LD_{50} (sodium benzoate)	1.90	1.72

*Average of 4.3 for males and 3.65 for females.

The LD_{50} values for free sorbic acid are considerably higher than those for the more soluble sodium sorbate. This indicates that sorbic acid when fed as such is less rapidly absorbed than is sodium sorbate. The average LD_{50} figure for sodium sorbate was found to be 4.0 g./kg. by Laboratory *A* and 5.94 g./kg. by Laboratory *B*. The variation is to be expected since different sized rats were employed; moreover, in one case, the material was given to fasted rats and in the other case to rats which had partaken

*Obtained from the South Charleston plant of Carbide and Carbon Chemicals Company.

†Obtained from the Matheson Co., East Rutherford, N. J.

of their food. On the other hand, the LD_{50} of sodium sorbate was almost twice that of sodium benzoate. The comparative ratios of LD_{50} of sorbate to benzoate reported by the two laboratories are quite consistent, namely 1.90 and 1.72.

Feeding tests on rats. It has been suggested *(8)* that fatty acids with conjugated double bonds might function in the animal organism as antimetabolites of the essential fatty acids. Thus, it is conceivable that elaeostearic acid, 9,11,13-octadecatrienoic acid, might act as an antagonist to linolenic acid, 9,12,15-octadecatrienoic acid. Moreover, sorbic acid, which has 2 conjugated double bonds, might be expected if the above hypothesis were true, to exert a similar action toward the essential dienoic acid, linoleic acid, which is 9,12-octadecadienoic acid.

However, there is considerable evidence that elaeostearic acid can be metabolized. Thus, Miller and Burr *(11)* reported that the trienoic acids in tung oil, which consists of approximately 80% of elaeostearic acid, are converted to diene acids which are gradually metabolized. Reiser *(16)* reports a similar metabolism of elaeostearic acids in hens with its deposition in the egg, while Fillerup and Mead *(5)* have demonstrated that methyl-β-elaeostearate is absorbed at the same rate as methyl oleate. In none of these tests was there any evidence of toxicity by the conjugated trienoic acid. Although these tests do not give information as to any possible deleterious action of elaeostearic acid over long periods of time, they do indicate that no immediate toxicity obtains and likewise that a trienoic acid can be converted to a dienoic acid which is eventually metabolized. These findings in themselves cast doubt upon the possibility that fatty acids with double bonds in congugated position are harmful.

In order to test whether or not sorbic acid or elaeostearic acid acts as an antimetabolite toward the essential fatty acids, these metabolites were incorporated at several levels in the fat-free diet employed earlier by Anisfeld *et al. (1)* to produce essential fatty acid deficiency. Rats receiving the fat-free diet without added sorbic acid or tung oil provided a control group. At the same time, records of food consumption and body weight were kept, from which data it is possible to calculate the efficiency of food utilization. The composition of the diets is recorded in Table 2, while the data on growth on the several diets are summarized in Table 3.

There is no evidence on the basis of the data recorded in Table 3 that the incorporation of sorbic acid or elaeostearic acid into a fat-free diet accelerates the rate of disappearance of the unsaturated fatty acids in the tissues and so shortens the depletion time for essential fatty acids. In fact, at 11 weeks, when depletion had occurred in the control group, there were no symptoms indicating that essential fatty acid depletion had occurred in the groups receiving sorbic acid or tung oil. Although Group 35c might appear to be depleted, the loss in weight was shown to be due to the failure of these rats to eat. When these rats were placed on the fat-free control diet, they continued to gain weight until the 19th week when the experiment was terminated.

The gains-in-weight of the rats receiving sorbic acid are identical, both at 6 weeks and at 11 weeks, with those of the rats on the control diet. Thus, after 11 weeks on the several diets, the average gains-in-weight were 137.0 $\pm$ 7.2 g., 152.0 $\pm$ 8.4 g., and 146.9 $\pm$ 6.3 g. for the rats receiving 0.5, 1.0, and 5.0% of sorbic acid respectively in the diets, as compared with a control value of 151.9 $\pm$ 4.3 g. Inasmuch as there was no relationship in weight gain to the sorbic acid content of the diet, the figures at 11 weeks are considered to be the same; the variations noted are due to the imprecisions of such biological tests. Actually, the values for the animals on the lowest (0.5%) sorbic acid diet may be regarded as constituting another control group.

The efficiencies with which the diets are utilized show no statistically significant differences. The average figures for efficiency ratio would seem to indicate less efficient utilization of the sorbic acid diets, if one were to disregard variability in responses of the animals. However, here also there is no tendency for the efficiency of food utilization (including the calories from sorbic acid) to decrease with an increasing content of sorbic acid in the diet. One could justifiably consider Group 38a which received 0.5% sorbic acid in the diet as another control group in evaluating the findings with animals on the 5% sorbic acid diet. On the basis of such a comparison, sorbic acid would necessarily be regarded as a source of calories in the diet. However, because of the small increment of calories which is contributed by sorbic acid, because of the *ad libitum* feeding, and because the growth response in relation to calories is on the upper plateau of the curve, the findings do not unequivocally prove that sorbic acid can

TABLE 2

Composition of the diets used in the growth tests with sorbic acid and with tung oil

LABORATORY A

Food component	Fat-free diet (39)	Sorbic acid diets			Tung oil diets		
		38a	38b	38c	35a	35b	35c
	%	%	%	%	%	%	%
Casein, vitamin test[a]	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Sucrose	71.62	70.75	69.88	62.92	71.02	70.42	65.62
Cellulose[b]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Salt mixture[c]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Sorbic acid[d]	....	0.50	1.0	5.0	....	....	....
Tung oil[e]	....	....	....	....	0.6	1.2	6.0
$NaHCO_3$[f]	....	0.37	0.74	3.70	....	....	....
Water-soluble vitamins[g]	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Fat-soluble vitamins[h]	....	....	....	....	....	....	....
Caloric value per 100 g.[i]	368.4	368.3	368.2	364.8	....	....	....

[a] General Biochemicals, Chagrin Falls, Ohio.
[b] Solka Floc, which is a cellulose flour.
[c] Salt mixture of Osborne-Mendel *(13)*.
[d] Purchased from Union Carbide and Chemicals Company.
[e] The sample of tung oil was supplied by The Best Foods, Inc. Spectrophotometric analysis gave a value of 87.8% elaeostearic acid, expressed on the total fatty acid content.
[f] Chemical equivalent of sorbic acid added.
[g] The water-soluble vitamins containing menadione were added in the following amounts per kg. of diet:

Thiamine	36 mg.	Folic acid	10 mg.
Riboflavin	72 mg.	Niacine	60 mg.
Pyridoxine	27 mg.	PABA	1,000 mg.
Calcium pantothenate	67 mg.	Inositol	500 mg.
Menadione	5 mg.	Choline	2,000 mg.
Biotin	1 mg.	Vitamin B_{12} (added in 95% ethyl alcohol)	0.01 mg.

The total is 3,778.01 mg./100 g. or 0.38%. Thiamine, riboflavin, pyridoxine, calcium pantothenate, niacin, choline, and vitamin B_{12} were kindly furnished by Merck and Co., Inc.; biotin was contributed by Hoffman-LaRoche, Inc.; and folic acid was a gift of the Lederle Laboratories.
[h] The fat-soluble vitamins A, D, and E were given orally once weekly. A total of 4 drops of the following mixture was given to each rat: 2.2 g. Nopco oil (800,000 U.S.P. units vitamin A and 80,000 U.S.P. units vitamin D per g.) and 51.5 g. of mixed tocopherols (340 mg. α-tocopherol per g.) made up to 200 ml. with propylene glycol.
[i] Based on a caloric value of 6.63 Cal./g. for sorbic acid *(12)*.

be utilized for growth as a source of calories. A more precise and direct approach is described in the second paper of this series *(2)*. In any case, the results reported here definitely indicate that sorbic acid does not interfere with the utilization of other foodstuffs for maintenance and growth.

Subacute toxicity studies of sorbic and benzoic acids on rats. The subacute toxicity tests were performed in Laboratory *B* in much the same way as the feeding tests described above except that the animals were sacrificed after 90 days on the diets and their tissues subjected to pathological examination.

The basal diet employed in these studies was a modified Food Research Laboratory diet 2C *(6)* consisting of dried whole milk, ground whole wheat, salt, yeast, and liver extract. It was freely available in McCollum-type food cups. Sorbic acid or sodium benzoate was fed at levels of 1, 2, 4, and 8% of the diet. One group subsisted on the diet without any additions, and one group on a 0.5% sorbic acid diet. Each group consisted of 5 male and 5 female rats picked at random from young rats raised in the Mellon Institute laboratory.

Tables 4 and 5 give the significant data for the groups receiving sorbic acid or sodium benzoate respectively. The level of statistical significance is indicated by the determination of the value for "p" for the "t" test for differences of each mean from that of the specific control group. When "p" is 0.05, the chance of the average being different from the control is one in 20. Only when the p-figures are 0.05 or less, are the results considered significant.

The growth tests with sorbic acid reported in Table 4 in Laboratory *B* confirm in every respect those carried out in Laboratory *A*, which are summarized in Table 3. Thus, the food consumption and gain-in-weight of the rats over the 90-day experi-

TABLE 3

The body weights of male rats receiving a fat-free diet or ones containing different levels of sorbic acid or tung oil as well as calculations of efficiency of utilization of the sorbic acid diets

LABORATORY A

Category	Groups receiving following diets						
	(Fat-free) 39	Sorbic acid tests			Tung oil tests		
		38a	38b	38c	35a	35b	35c
Number of rats	20	10	10	10	10	10	10
Test material in diet, %	....	0.5	1.0	5.0	0.6	1.2	6.0
Average body weight, g.							
Start	36	34	34	36	35	34	36
After 3 weeks	96	98	100	98	99	101	95
After 6 weeks	154	146	155	154	158	165	157
After 9 weeks	186	164	172	171	189	188	179
After 11 weeks	189	171	186	183	212	201	172
After 19 weeks[a]	217	206	221	233	241	254	263
Average gain-in-weight, g.[b]							
After 6 weeks	117.8±3.4	112.0±6.9	120.4±8.2	118.2±3.6	....	....	....
After 11 weeks	151.9±4.3	137.0±7.2	152.0±8.4	146.9±6.3	....	....	....
Average food consumed, g.							
After 6 weeks	436.6	455.5	485.9	497.4	....	....	....
After 11 weeks	949.0	907.2	966.7	987.9	....	....	....
Average food efficiency[b, c]							
After 6 weeks	7.30±0.17	6.67±0.22	6.69±0.26	6.56±0.19	....	....	....
After 11 weeks	4.33±0.12	4.19±0.22	4.25±0.13	4.08±0.17	....	....	....
Average total sorbic acid eaten, g.	0	4.53	9.67	49.40	....	....	....

[a] Rats received fat-free diet starting on 12th week.

[b] Including Standard Error of Mean calculated from the formula $\sqrt{\Sigma d^2/n-1}/\sqrt{n}$ where "n" is the number of observations and "d" is the deviation from the mean.

[c] Calculated by dividing the gain in body weight × 100 by the calories consumed.

TABLE 4

The results of the 90-day feeding tests on rats receiving diets containing different levels of sorbic acids or a sorbic acid-free control diet

LABORATORY B

Category	Sorbic acid-free control diet	Diets containing sorbic acid in %				
		0.5	1.0	2.0	4.0	8.0
Sorbic acid dosage, g./kg./day	0.0	0.32	0.63	1.26	2.48	5.06
Diet consumed, g./rat/day	11.97	12.57	12.62	11.98	12.08	12.36
p for t—test	....	0.65	0.55	0.99	0.93	0.72
Av. weight gain of surviving rats, g.	156.3	164.4	169.0	161.8	190.1	158.6
p for t—test	....	0.76	0.60	0.84	0.29	0.94
Liver weight, % body weight	3.17	3.20	3.27	3.25	3.52	3.54*
p for t—test	....	0.83	0.52	0.51	0.06	0.04
Kidney weight, % body weight	0.64	0.63	0.64	0.62	0.65	0.65
p for t—test	....	0.65	1.00	0.24	0.73	0.65
Uninfected rats**	9	9	9	9	7	7
Toxic deaths	0	0	0	0	0	0
Sets of rat tissue with any pathology	0	0	0	0	0	0

* Results statistically significant when compared with control.

** Tissues examined from all these rats.

mental period in the case of all groups receiving sorbic acid (including at an 8% level) are not different from those of the control group. Moreover, all tissues which were examined of all the rats on the several sorbic acid regimens were normal. There was a slight increase in liver weights as percentages of the body weights in the group receiving 8% sorbic acid (average liver weight being 3.54% of body weight) when compared with those on the control diet (3.17% of body weight). Although this figure for the 8% sorbic acid group is significantly higher than that of the control ($p = 0.04$, barely over the usually accepted level of significance), even in this case it must be considered as representing merely an extremely mild enlargement of normal liver; no micropathology of the liver was evident at any level of sorbic acid fed. The percentage of body

TABLE 5

The results of the 90-day feeding tests on rats receiving diets containing different levels of sodium benzoate or a sodium benzoate-free control diet

LABORATORY *B*

Category	Sodium benzoate-free control diet	Diets containing sodium benzoate in %			
		1.0	2.0	4.0	8.0
Sodium benzoate dosage, g./kg./day	0.0	0.64	1.32	2.62	6.29
Diet consumed, g./rat/day	11.97	12.69	12.63	12.30	11.98
p for t—test	....	0.54	0.62	0.75	0.99
Average weight gain of surviving rats, g.	156.3	170.7	160.5	150.0	100.2
p for t—test	....	0.58	0.86	0.82	0.06
Liver weight, % body weight	3.17	3.01	2.97	3.12	4.46*
p for t—test	....	0.26	0.26	0.89	0.0005
Kidney weight, % body weight	0.64	0.64	0.65	0.70	0.90*
p for t—test	....	1.00	0.65	0.06	0.0001
Uninfected rats	9	9	10	8	8
Toxic deaths	0	0	0	0	4
Average number days to death	....	....	....	....	13.0
Sets of tissues examined from uninfected rats	9	9	10	8	7
Sets with major pathology	0	0	0	0	1
Sets with any pathology	0	1	1	1	7

* Results statistically significant as compared with control.

weight represented by the weight of the liver has been reported in other studies *(4)* to be as high as 5.34% after a choline-free diet containing butter fat which was administered for only 16 days, in contrast to a value of 3.17% when choline was added to the regimen. In Laboratory *B*, no change in kidney weight was observed, as related to the sorbic acid intake and no histopathological changes in that organ. It may be concluded that sorbic acid when incorporated in the diet at a level as high as 4% has no harmful effect on rats; at 8%, it is highly doubtful that any toxicity occurs within 90 days.

The results obtained in the rats receiving sodium benzoate are in striking contrast to those noted in the sorbic acid tests. When this substance was present in the diet at the 8% level, the gain-in-weight of the *survivors* in the group over the 90-day period was only two-thirds of that of the control rats on an identical food intake. Statistical analysis of the growth responses indicated that this was just below the usually accepted level of significance ($p = 0.06$). This interpretation of a non-significant difference does not mean that a real difference does not exist; the limited number of survivors, 4 out of the original group of 8, resulted in the evaluation of such a limited number of responses that differences would have to be enormous to be significant. Furthermore, the weights of both the livers and kidneys of the rats in this group were significantly higher ($p = 0.0005$ and 0.0001, respectively) than were those of the control group. The weights of these organs were 140% of those of the control rats. Four rats died without infections during the study, and one is forced to conclude that death was caused by the agents fed. Moreover, frequent pathological lesions were noted in the

livers and kidneys of the group which received 8% of sodium benzoate, although they were seldom severe.

At the 3 lower levels of benzoate feeding (4, 2, and 1%) one liver in each case was found with light cloudy swelling among the uninfected survivors of the several groups. Although this degree of pathological lesions was not found in the specific controls of the study, it is not an infrequent finding in control animals and probably should be considered as an artifact. If one accepts such an assumption, one is justified in concluding that 4% of sodium benzoate in the diet is harmless under the conditions of the test.

On the basis of these subacute toxicity tests, one may state that sorbic acid and sodium benzoate when fed at a level of 4% in the diet have no harmful effect on rats in a 90-day feeding study. Sorbic acid at 8% is probably harmless while sodium benzoate at an 8% level is definitely toxic.

Subacute toxicity tests of sorbic and caproic acids on dogs. The tests on dogs were designed to determine whether or not sorbic acid exerts any harmful effects when fed as the free acid to puppies over a 3-month period following weaning. Two sets of controls were carried out, *viz.*, one in which the dogs received a comparable dose of caproic acid to that of sorbic acid and the second in which the dogs received only the basal diet.

Nine puppies from 3 litters were purchased commercially. The dogs from each litter were distributed between the 3 dietary regimens. In the case of litter 2, which comprised 4 dogs, 2 were placed on the sorbic acid regimen. The dogs ate all 3 diets with gusto and only on several occasions were the diets not completely consumed. The animals were kept in separate cages for the duration of the test except that they were exercised in a large yard for one-hour periods each morning and afternoon. The dogs were fed once daily. Daily records were kept of their body weights. After the animals had been on several diets for 90 days, they were sacrificed, blood samples were obtained for hemoglobin determinations and the following organs were removed: liver, kidney, heart, lung, pancreas, stomach, small intestine, spleen, adrenal glands, thyroid gland, parathyroid gland, gonad, striated muscle and skin. The tissues and/or organs from each animal were placed in 10% formalin fixative in glass jars and submitted to the pathologist[5] for histopathological examination with a code number known only to the laboratory worker submitting the samples.

The composition of the several diets is given in Table 6. The presence of sorbic or caproic acids in the diets was confirmed by the analyses carried out in the laboratory of The Best Foods, Inc. of samples forwarded to them; these analyses are recorded in Table 7. Table 8 gives the data on the food consumption and weight gains in the dogs.

The animals seemed uniformly healthy, vigorous, and frisky during the experimental period except for a short interval when Dog 36 showed the presence of coccidiosis in the stools. The infection had been present in dogs previously purchased which had been discarded before being used for experiment. None of the other dogs employed in the present study became infected and Dog 36 made a quick recovery. At no time during the experimental period were there any additions or subtractions from the diet.

With the exception of Dog 36 which had lost considerable weight during the period when he had coccidiosis, the gain-in-weight of the animals in any one litter on the several dietary regimens was fairly uniform. For example, in litter II, both dogs on the sorbic diet gained more than the caproate control but slightly less than the dog on the control diet. In the other 2 litters, the dogs receiving the caproate diet did as well as those on the control diet.

The level of hemoglobin at the termination of the tests was satisfactory in all cases. The values averaged as follows: control diet, 11.4% (3 dogs); caproic acid diet, 13.6% (3 dogs); and sorbic acid, 12.2% (2 dogs). Although some minor pathological changes were noted in the tissues, no alteration was found to occur repeatedly, with any significance, in any one group. It was therefore concluded that sorbic acid in an amount of 4% (5% on a moisture-free basis) in the diet was harmless to dogs. This conclusion was based on the fact that the animals remained in excellent condition throughout, by the lack of any specific abnormalities which could be detected on gross examination of

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[5] We are indebted to Dr. H. Russell Fisher, Professor of Pathology, University of Southern California School of Medicine, for the histopathological examinations.

TABLE 6

Composition of the diets used in the growth tests of dogs with basal diet alone or with basal diet containing sorbic or caproic acid [a]

LABORATORY A

Food component	Basal diet (1)	Basal diet containing caproic acid (2)	Basal diet containing sorbic acid (3)
	%	%	%
Cheddar cheese (mild) [b]	50.0	50.0	50.0
Margarine fat	2.0	0.0	0.0
Cod-liver oil	5.0	5.0	5.0
Sucrose	18.5	16.5	16.5
Dextrin	15.0	15.0	15.0
Cellu flour [c]	2.0	2.0	2.0
Salt mixture [d]	1.5	1.5	1.5
Dried brewer's yeast [e]	5.0	5.0	5.0
Liver extract [f]	1.0	1.0	1.0
Caproic acid	0.0	4.0	0.0
Sorbic acid	0.0	0.0	4.0

[a] The control basal diet contains 20% of moisture Diets 2 and 3, containing caproic and sorbic acid, respectively, involve an iso-caloric substitution. The diets are prepared as follows: To the cheese, melted to a flowable mass, are added without further heating the cod-liver oil and the melted margarine fat, caproic acid, or sorbic acid, as the case may be. The mixed dry ingredients are then added, and the blended mixture stored in the refrigerator. The chilled product was broken into small bite-size granules prior to feeding. Diets were kept at 45°F. for not longer than 2 weeks.

[b] Purchased in 25-lb. wheels from the Challenge Dairies, Los Angeles, Calif.

[c] Solka Floc which is a cellulose flour.

[d] Wesson modification *(18)* of salt mixture of Osborne-Mendel *(18)*.

[e] Anheuser-Busch, Strain G.

[f] Wilson, 1:20 liver concentrate.

TABLE 7

The analysis of samples of diets used in dog experiments for sorbic acid and total fat [a]

LABORATORY A

Category	Fresh (F) or week-old diet (O)	Basal diet (1)	Basal diet containing caproic acid (2)	Basal diet containing sorbic acid (3)
pH of 10% suspension in distilled water	F	5.9	4.8	5.0
	O	5.9	4.8	5.0
Sorbic acid, % [b]	F	....	....	4.2
	O	....	....	4.4
Fat, %	F	22.8	24.7	24.8
Analysis of fat:				
Acid value [c]	F	0.4	130.4	125.3
	O	0.7	130.7	125.7
Peroxide value [d]	F	5.9	6.0	6.1
	O	13.2	13.4	16.5
Thiobarbituric acid reaction, [e] ($E^{1\%}_{1cm}$, at 535 mμ)	F	0.086	0.091	0.092
	O	0.108	0.127	0.126

[a] Analyses conducted in the laboratory of The Best Foods, Inc. The diets were analyzed as received and after one week at 75°F.

[b] Analysis by spectrophotometric method described in *(10)*.

[c] Mgs. KOH per g. sample.

[d] Milliequivalents per kg.

[e] A measure of aldehydic oxidation products (fat basis) which react with 2-thiobarbituric acid to give a pink color with maximum absorbency at 535 mμ under conditions of the test *(14)*.

TABLE 8

Summary of feeding tests on dogs

LABORATORY A

Litter and dog number	Description of litter	Dietary regimen[a]	Sex	Duration of feeding test	Weight in grams			Hemoglobin at end
					Start	End	Gain	
				days				%
I, 34	Mixed cocker and terrier	1	F	91	1800	5100	3300	14.0
57		2	M	91	2425	5870	3445	13.8
36		3	M	89	2300	4430	2130	....[b]
II, 50	Half cocker	1	M	88	1407	4360	2853	10.3
38		2	M	88	1365	3480	2015	14.0
33		3	F	88	1700	4370	2670	13.0
59		3	M	88	1680	4000	2320	11.5
III, 37	Half cocker	1	M	90	2045	5800	3755	10.0
2		2	M	90	2145	6300	4155	13.0

[a] Diet 1 is control diet; Diet 2 contains 4% caproic acid (wet basis); and Diet 3 contains 4% sorbic acid (wet basis).

[b] Dog 1c (No. 36) was run over and killed on 89th day. Animal had had coccidiosis from 20th to 25th day but had recovered.

the organs of the animals when they were sacrificed at the end of the feeding experiments, and by the report of the histopathological examination of the tissues which showed no abnormal conditions.

SUMMARY

1. The results of the present experiments indicate that sorbic acid is harmless to rats and to dogs when incorporated in the diets to the extent of 5% (moisture-free basis). This conclusion is based on the following experimental findings:

a. In tests with 2 different strains of rats conducted in 2 separate laboratories, the gain-in-weight of rats receiving diets containing up to 8% of sorbic acid was similar to that of rats on the basal diet free of sorbic acid. Similar results were obtained in one series of tests involving supplementation of a fat-free diet with 5% sorbic acid.

b. There was no evidence of abnormality in liver or kidney weights or of pathological conditions in other tissues of the rats receiving 4% of sorbate. Rats on the 8% sorbic acid diet exhibited a slight but statistically significant increase in liver weight, expressed as percentage of body weight, but this represented merely an extremely mild enlargement of liver without micropathology.

c. Sorbic acid does not act as an antimetabolite for essential fatty acids in rats. It was likewise found that elaeostearic acid did not act as an antagonist to the essential acids.

d. The incorporation of sorbic acid into the diet did not decrease the utilization of the foodstuffs fed concomitantly, as determined from the efficiency ratio (gain-in-weight × 100) : calories consumed. Indeed, the present findings would seem to indicate that sorbic acid is utilizable by the animal organism as a source of calories. The results of metabolic studies to be reported have confirmed this interpretation.

e. In tests with dogs, similar response was obtained when the animals received a 50% Cheddar cheese diet with other food components alone, or

the same diet with 4% sorbic acid or 4% caproic acid incorporated as the free acid. A histopathological examination of tissues at the termination of the tests failed to indicate any pathological lesions attributable to the experimental diets.

2. The results of the present experiments indicate also that sorbic acid is far less toxic than sodium benzoate. This conclusion is based upon the following experimental findings:

a. Sorbic acid has been shown in 2 independent laboratories to have a relatively high LD_{50}. When fed as a free sorbic acid to rats, in one laboratory, the LD_{50} was found to be 10.50 g./kg. When fed as sodium sorbate to rats having access to food, the LD_{50} was found to be 5.94 g./kg. (calculated as sorbic acid); on the other hand, when fed to fasted rats, the LD_{50} was found to be 4.3 g./kg. for male rats and 3.65 g./kg. for female rats.

b. Sodium benzoate was found to have an LD^{50} of 3.45 g./kg. in one laboratory with rats having access to food, and 2.1 g./kg. in another laboratory with fasted rats. The ratios of LD_{50} (sodium sorbate) : LD_{50} (sodium benzoate) based on calculations as the free acids, were found to be 1.72 and 1.90 in the two laboratories.

c. Unlike sorbic acid, sodium benzoate was definitely toxic to rats when fed at a level of 8% of the diet. Growth and survival were adversely affected and histopathological changes in liver and kidney occurred.

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STUDIES ON AMINO ACID METABOLISM. III. PLASMA GLYCINE CONCENTRATION AND HIPPURIC ACID FORMATION FOLLOWING THE INGESTION OF BENZOATE[1]

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(Received for publication January 16, 1948)

The conjugation of benzoic acid with glycine, resulting in the formation of hippuric acid, takes place in liver (1) and kidney (2). The urinary output of this conjugate after the administration of benzoate is used to measure this aspect of hepatic function (3).

Theoretically, decreased hippuric acid formation may be due to hepatic and/or renal disease or to an inadequate supply of glycine. In this regard it is not clear to what extent this amino acid is available as such in the liver or is formed *ad hoc*, either in this organ or elsewhere. The present investigation was undertaken in the hope that data bearing on this question might be obtained.

PROCEDURE AND METHODS

Six or 10 gms. of sodium benzoate[2] were given orally to fasting normal or diseased subjects, who were instructed to drink approximately 500 cc. of water. The urine collected during the subsequent three hours was analyzed for its hippuric acid content according to the method of Quick (5). Fasting venous blood was obtained just before the ingestion of benzoate and at intervals thereafter. In some cases an additional fasting sample was taken one-half or one hour before the administration of benzoate. Plasma glycine was determined by the method of Alexander *et al.* (6). The influence of orally administered glycine was also studied in a few cases.

RESULTS

Benzoate was administered without additional glycine 11 times to ten individuals (Table I), and in all instances except one (No. 11) a significant decrease in plasma glycine occurred. In some cases the decline began within ¾ hour after ingestion and continued thereafter (Nos. 1, 2, 7, 8, 9). In others it remained at the low level reached within one hour (Nos. 3, 4, 10); in two subjects (Nos. 5, 6) it started to rise again during the period of observation. The largest drop in plasma glycine amounted to 2.07 mgm. per cent (5% per cent of the initial concentration); in general, however, the extent of the drop was between 20 and 35 per cent of the fasting glycine concentration, and was unrelated to this value.

The amounts of hippuric acid excreted varied widely. It is seen that in the only case in which no decrease in plasma glycine occurred, the hippuric acid excretion was definitely subnormal (1.9% gms., as compared with a minimal normal excretion of 3 gms.) It appears, however, from a comparison of cases 9 and 11, and 2 and 5, that no positive correlation exists between the quantity of excreted hippuric acid and the decrease in plasma glycine, although admittedly the number of observations is too limited to permit drawing definite conclusions.

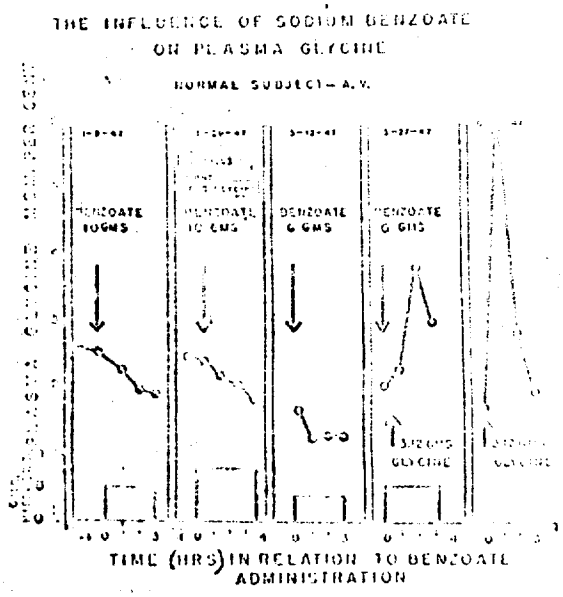

Fig. 1.

[1] Supported by a grant from the John and Mary Markle Foundation, New York City.

[2] Sodium benzoate was used in preference to benzoic acid because of its more rapid absorption from the intestinal tract (4).

Five experiments were performed on one normal subject (Nos. 2, 3, 12, 13, 13a). The data are presented in Figure 1. The ingestion of 10 gms. of benzoate resulted in a larger hippuric acid excretion and a greater absolute drop in plasma glycine than the administration of 6 gms. Furthermore, the ingestion of 10 gms. of glycine the night prior to the test resulted in a larger hippuric acid excretion, without, however, a greater decline in plasma glycine than obtained following the administration of 10 gms. of benzoate alone. The administration of glycine half an hour after the

TABLE 1

The effect of benzoate on plasma glycine and on hippuric acid formation

	No.	Sub.	Sex	Age	Diagnosis	Na benz. taken	Hipp. acid excretion		Urine vol.
						gms.	*gms.*	*hrs.*	*cc.*
No supplementary glycine	1	M. G.	F	21	Bronchial asthma	10			
	2	A. V.	M	35	Normal (1-9-47)	10	5.10	3	440
	3	A. V.	M	35	Normal (5-12-47)	6	3.77	3	360
	4	F. B.	F	44	Essential hypertension	10			
	5	W. T.	M	60	Arteriosclerosis; heart disease, congestive failure	6	3.48	3	355
	6	L. S.	M	70	Infectious hepatitis	6	4.15	3	950
	7	I. G.	M	43	Infectious hepatitis	6	4.28	3	[illegible]
	8	S. R.	M	49	Ca. of pancreas	6	4.2	3	[illegible]
	9	E. A.	F	32	Nutrit. hypoproteinemia	6	2.03	4	[illegible]
	10	Th. W.	M	52	Virus pneumonia; malnutrition. Reconvalescent (5-8-47)	6	2.61	3	[illegible]
	11	P. J.	M	25	Rheumatoid arthritis	6	1.92	3	300
Additional glycine given*	12	A. V.	M	35	Normal (1-29-47); 10 gms. glycine taken night before test	10	7.69	33	[illegible]
	13	A. V.	M	35	Normal (5-27-47); 3.12 gms. glycine taken ½ hour after ingestion of 6 gms. Na benzoate	6	5.18	33	[illegible]
	13a	A. V.	M	35	Normal (6-2-47); 3.12 gms. glycine w/o Na benzoate				
	14	Th. W.	M	52	Virus pneumonia; malnutrit. Reconvalescent. 3.12 gms. glycine taken simultaneously w/o gms. Na benzoate	6	4.92	3	610

TABLE 1—*Continued*

	No.	Sub.	Plasma glycine—*mgm. per cent* Fasting					Plasma glycine—*mgm. per cent* After benzoate ingestion													Plasma glycine decrease	
			Time in minutes					Time in minutes														
			−60	−45	−30	−15	0	15	30	45	60	75	90	105	120	135	150	165	180	195	*mgm. per cent*	*per cent*
No supplementary glycine	1	M. G.		2.66			2.47			1.82				1.65							0.8[illegible]	33
	2	A. V.	2.61				2.56		2.27				1.99				1.91				0.65	[illegible]
	3	A. V.					1.65				1.27				1.33				1.30		0.[illegible]	[illegible]
	4	F. B.					2.06			1.56				1.47				1.48			0.5[illegible]	[illegible]
	5	W. T.					2.10				1.37				1.15				1.42		0.6[illegible]	[illegible]
	6	L. S.	2.19				2.17			1.70									1.91		0.2[illegible]	[illegible]
	7	I. G.			1.91		1.92				1.61				1.61				1.4[illegible]		0.3[illegible]	[illegible]
	8	S. R.		1.99			1.85		1.75						1.45						0.4[illegible]	[illegible]
	9	E. A.			3.86		3.90		2.81						2.17					1.86	2.0[illegible]	52
	10	Th. W.		1.44			1.33				0.92				0.85				0.2[illegible]		0.4[illegible]	[illegible]
	11	P. J.			1.26		1.29				1.22				1.20				1.28		0	[illegible]
Additional glycine given*	12	A. V.		2.49			2.41				2.19				2.10				1.8[illegible]		0.5[illegible]	[illegible]
	13	A. V.					2.02		†		2.31				3.80				2.00			
	13a	A. V.					1.69†		5.81				2.78				1.91					
	14	Th. W.					1.73†				2.89				2.60				2.60			

* Glycine administered orally. † Time of glycine ingestion.

benzoate ingestion in an amount equivalent (chemically) to the benzoate ingested also increased the hippuric acid output; plasma glycine rose, but to a lesser extent than when glycine alone was administered.

The influence of additional glycine on hippuric acid formation was also apparent in subject Th. W. (compare No. 10 with No. 14 of the table).

DISCUSSION

Leuthardt and Glasson (7) and Quick (1) assumed that the glycine needed for the conjugation of benzoic acid is synthesized by the liver at a rate which is the principal limiting factor in hippuric acid synthesis. It has been known (1, 8-11) and reconfirmed above that the administration of glycine in addition to benzoate increases hippuric acid formation. In senile individuals, subnormal hippuric acid formation may be due to inability of the liver to furnish enough glycine since supplements of this amino acid restore hippurate excretion to normal (12).

That stores of free glycine are present in the body has been shown (13, 14). The drop in plasma glycine occurring after benzoate administration, first reported by Christensen (14) and confirmed by us, indicates that these stores are utilized in hippuric acid formation probably faster than the amino acid can be synthesized or mobilized. The question arises if and to what extent the concentration of plasma glycine influences the synthesis of hippuric acid. The subnormal hippuric acid output and absence of plasma glycine drop in Case No. 11, whose fasting plasma glycine was extremely low, can be interpreted in two ways: either not enough glycine, as reflected by the low plasma glycine concentration, was available for hippuric acid formation, or a primary disturbance in liver function of this patient prevented glycine-benzoate conjugation. In accord with the latter concept is the decline of plasma glycine in Case No. 10, whose fasting plasma glycine was also subnormal.

It appears that an elevated blood glycine concentration consequent to glycine ingestion (compare No. 3 with 13, 11, and with 14) is accompanied by increased hippuric acid formation. A high plasma glycine level per se, however, does not necessarily result in a very large hippuric acid output even though a considerable drop in plasma glycine may occur after benzoate ingestion (No. 9). It would thus seem that the concentration of plasma glycine cannot be considered an absolute measure of the total amount of glycine available for uptake by the liver (and kidney?). The net decrease in plasma glycine following benzoate administration may be influenced not only by uptake by the liver but also by other factors such as plasma volume, and by the rate of replacement of plasma glycine by tissue stores of the amino acid. In this respect it is noteworthy that the subject of Experiment 9 had an exceptionally low body weight and, accordingly probably a low plasma volume. Furthermore, an intravenous glycine tolerance test on this subject (reported elsewhere [15]) indicated a slow equilibration of plasma glycine with the tissues. It should be pointed out, however, that because of the low body weight 6 gms. of benzoate in this patient would be comparable to a much larger dose in heavier subjects.

That glycine given simultaneously with benzoate is utilized for hippuric acid synthesis is indicated not only by increased hippuric acid formation but also by a smaller rise in plasma glycine than when the same amount of amino acid is given alone. On the other hand, the rise which did occur, even when glycine was simultaneously administered in amounts *chemically equivalent* to the ingested benzoate, indicates that part of the ingested glycine was not utilized during the observation period. This is probably attributable to a faster rate of glycine absorption than benzoic-glycine conjugation, whatever the limiting factors of the latter may be.

The large hippurate excretion observed when glycine was taken the night prior to benzoate suggests that glycine was stored in the liver (and kidney?) either in free, or in otherwise readily available form. The latter is more probable since it is known that three hours after glycine injection the concentration of easily extractable amino acids in the liver has returned to normal (16).

From the data it appears that within certain limits the rate of conjugation of benzoate with glycine depends to some extent upon both the dose of benzoate and the availability of glycine in the body. Under certain conditions the latter may be significantly curtailed. Accordingly, to obviate these factors in hippuric acid studies of liver function, it might be advantageous to administer

the benzoate in doses determined by the total body weight or surface area and to give the amino acid before or with the benzoate in at least a chemically equivalent amount.

CONCLUSIONS

1. In ten out of 11 experiments the administration of sodium benzoate resulted in a decrease in plasma glycine concentration. In one case with subnormal hippuric acid excretion the plasma glycine remained unaltered.

2. The administration of glycine before or together with the benzoate results in a larger output of hippuric acid than when benzoate alone is ingested.

3. The rate of conjugation of glycine to benzoate, the availability of glycine for this purpose, and the size of the dose of benzoate, are important factors which affect hippuric acid synthesis in man.

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UDC 616-006.4-02:664.8.035-092.9

EFFECT OF CERTAIN FOOD PRESERVATIVES ON THE DEVELOPMENT OF MALIGNANT PROCESSES IN MICE

(Vliyanie nekotorykh pishchevykh konservantov na razvitie zlokachestvennogo protsessa u myshei)

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There has been a sharp increase in the use of various chemicals in the food industry. We possess only scant knowledge of the possible toxic and carcinogenic effects of these additives to food products when they penetrate the human organism for prolonged periods of time.

Sodium bisulfite is used in the treatment of fruit, vegetables, and small berries for the prevention of mold. Its permissible residual concentration is up to 300 mg/1 kg. Benzoic acid is used mainly in the treatment of the same products and their preserves in order to prevent fermentation; its concentration does not exceed 0.2% of the product by weight. Both preservatives have been in use for about half a century.

Until recently, sodium bisulfite and benzoic acid were regarded as relatively harmless noncarcinogenic substances (Kiechebusch and Lang; Hosino; Lockett and Natoff). Recently, however, several authors have demonstrated some unfavorable effects of these substances on animals (Fitzhugh et al.; Morgan); the use of benzoic acid has therefore been prohibited in certain countries (France), and the use of sulfur compounds is under discussion at the World Health Organization.

Sorbic acid and nisin are new preservatives. Sorbic acid, originally isolated from rowan, proved to be a very efficient preservative. This is a polyunsaturated fatty acid with six carbon atoms. According to the literature (Lang), addition of 5% sorbic acid to the ration of rats did not cause any pathological changes, while addition of 8% of the same substance (Deuel et al.) increased the weight of the liver. We did not find any reports of carcinogenic properties of sorbic acid in the literature.

The antibiotic nisin was originally isolated from acid dairy products. This is a product of vital activity of lactic streptococci and has no medical value, but it lowers the resistance of spores of thermally stable bacteria to heating, so that its addition to preserves permits lower sterilization temperatures, thus improving the food value of the preserves. There are no data on carcinogenic properties of nisin.

Studies of the toxicity of benzoic acid in mice in concentrations used for human food products gave results as follows: the survival time of the mice was the lowest recorded in this series of experiments; the gain in weight was the lowest; there was impairment in the detoxifying function of the liver, and the susceptibility of the animals to the unfavorable results of cold and centrifugation (Ignat'ev) was highest.

The combined use of benzoic acid and sodium bisulfite also resulted in a markedly reduced survival time of mice, a decrease in their consumption of water and food, lag in weight gain and lowering of resistance to physical loads and starvation. There was also a hypertrophy of certain organs, especially the liver.

Sorbic acid proved to be nontoxic. Its inclusion in the animals' rations resulted in a higher survival time, more rapid increase in weight, greater consumption of food, greater phagocytic activity in comparison to the control animals, etc.

Nisin possessed the highest toxicity, alone or when mixed with sorbic acid. At the same time there was a certain antagonism in the effects of sorbic acid and nisin. On the whole, all the indices mentioned above were nearer to normal values than in the case of nisin alone, probably owing to the favorable effect of sorbic acid.

In all experiments in which mice received nisin alone or nisin in combination with sorbic acid their gain in weight was greater. In a certain series of experiments the animals were fat, the body weight exceeding 40 g in 44% of the experimental animals.

There were also cases of adiposity among animals which received sodium bisulfite in combination with benzoic acid, mainly in females (Ignat'ev).

In our experiments we used 365 mice of both sexes. They were divided into five groups, four experimental and one control. All animals received identical rations, first of natural products (according to standards established by the Academy of Medical Sciences of the USSR), later briquetted food. Experimental mice received an addition of the preservatives being investigated, 0.2% benzoic acid, 0.01% nisin, 0.2% sorbic acid or 0.4% sodium bisulfite (percentages by weight of the total rations). The doses of preservatives were the same as those used in foodstuffs for human consumption. Special groups of mice were used for studies of the combined effects of the mixtures of sodium bisulfite + benzoic acid and sorbic acid + nisin. For three months the experimental animals received these preservatives in their food in the amounts indicated above, and this was followed by intraperitoneal inoculation with Ehrlich's ascites carcinoma to the experimental and control animals.

As is known, the introduction of 50—80 million tumor cells into the abdominal cavity of mice causes the development of Ehrlich's ascites carcinoma in 100% of the experimental animals, and their survival time after the inoculation is 12 to 17 days. According to our preliminary observations, the introduction of a smaller number of tumor cells (1.2 - 1.25 million cells) led to the development of tumors in 70—100% of mice, and their survival after the inoculation was somewhat prolonged (21—30 days).

This was the method of inoculation used by us in order to provide a more distinct expression of the effect of the preservative on the development of ascites carcinoma. The mice were weighed before the experiment and their abdominal volume was measured. After this, measurements were performed at intervals of 4 days, beginning with the day of transplantation of the tumor and until the animals died. The observation period was 53 [illegible] days, and then all the surviving mice were killed and autopsied. All the mice (those which died spontaneously or were killed after the termination of the experiment) were measured for the amount of ascitic fluid and its blood content. The latter was designated in arbitrary units as follows: weak pink color — 1 unit, red color — 2 units, and dark red color — 3 units. The results were statistically analyzed and the P value was determined by comparing the indices of a given group against the control.

The results are listed in Tables 1 and 2.

It will be seen from Tables 1 and 2 that the successful inoculation with tumors was more frequent in mice which received sodium bisulfite and benzoic acid on their own and less so in mice which received both substances together. The shortest survival time after transplantation of the tumor was found in mice which received sodium bisulfite and benzoic acid on their own. The volume of ascitic fluid was greatest in mice which received sodium bisulfite. The percentage of mice which developed tumors among those receiving sorbic acid on their own or together with nisin did not differ from the percentage in control mice which did not receive additions of preservatives. The survival time of these mice after the transplantation was slightly shorter than in the control mice, and the volume of ascitic fluid in mice receiving sorbic acid was the same as in the controls; it

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Received 8 July 1964

SUMMARY

Effect of Certain Food Conservants on the Development of Malignant Processes in Mice

A.A. Dinerman, V.D. Ignatiev

In tests performed over 365 outbred mice of both sex was studied the effect of certain food conservants (sodium bisulfite, benzoic acid, sorbic acid, nizine) on the development of ascetic Erlich cancer. Those mice, that received sodium bisulfite and benzoic acid, presented a higher percentage of developed tumours, a shorter life length and, a larger amount of ascetic fluid than the mice, receiving sorbic acid and nizine as well as the control animals. The fattening caused by the conservants has no effect on the development of the malignant processes.

The same results were also attained with respect to the development of spontaneous hepatoma in male mice of the strain C_3H.

In our experiments, 90 mice were given either a mixture of sorbic acid + nisin or a mixture of sodium bisulfite + benzoic acid in doses indicated above after a period of 8 months. Adiposity incidence (percentages) differed according to the sex (occurring more frequently in females), and therefore our experiment included both males and females. The experiments included both adipose mice and mice of ordinary weight. For this purpose, the mice were divided into 9 groups, 10 animals in each group (males and females or ordinary weight, males and females of excessive weight, and the control group).

The experiment was designed to provide an answer to the question of whether the addition of the preservatives to the rations facilitated a more intensive development of the malignant tumor or whether this effect was secondary, coming as a corollary of adiposity due to the preservative used in the experiment.

All mice were given intraperitoneal doses of 1.2 – 1.25 million cells of Ehrlich's ascites carcinoma. We did not succeed in observing any difference in the development of the tumors in mice of the different groups, yet the survival time of the animals and the volume of the ascitic fluid were not identical. Thus, the survival time after transplantation of the tumor was somewhat shorter (11 – 13 days) in mice treated with the mixture of sodium bisulfite and benzoic acid, in comparison to the other groups, while the volume of their ascitic fluid was somewhat larger (9 – 11 ml), as against 15 days and 7 – 8 ml, respectively, in the control. The difference was statistically insignificant, but the trend described a higher malignancy of Ehrlich's ascites carcinoma in mice treated with sodium bisulfite in combination with benzoic acid. This effect is obviously unrelated to the capacity of these preservatives for causing adiposity.

The time at which the mice began to die, indicating the intensity of development of the tumor, the volume of ascitic fluid and its blood content are shown in Table 2.

Conclusions

1. When Ehrlich's ascites carciroma was transplanted to mice which received the older preservatives sodium bisulfite and benzoic acid with their food, the percentage of tumors which developed was the greatest. The survival time of these mice was the shortest, while the volume of ascitic fluid was the largest. These data indicate a higher malignancy of the tumors which developed in comparison to control mice.

2. The percentage of mice which developed tumors among those treated with sorbic acid and nisin, as well as the degree of malignancy of the tumors, was the same as in the control mice.

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SUMMARY

Effect of Certain Food Conservants on the Development of Malignant Processes in Mice

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In tests performed over 365 mongrel mice of both sex was studied the effect of certain food conservants (sodium bisulfite, benzoic acid, sorbic acid, nizine) on the development of ascetic Erlich cancer. Those mice, that received sodium bisulfite and benzoic acid, presented a higher percentage of developed tumours, a shorter life length and, a larger amount of ascetic fluid than the mice, receiving sorbic acid and nizine as well as the control animals. The fattening caused by the conservants had no effect on the development of the malignant processes.

Arch. int. Pharmacodyn., 1963, 144, No 1-2.

EXPERIMENTAL THERAPEUTICS RESEARCH SECTION LEDERLE LABORATORIES
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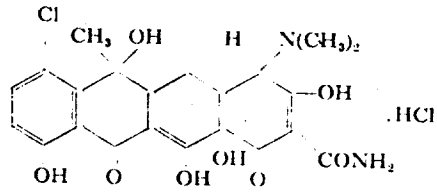
RELATIVE TOXICITY OF CHLORTETRACYCLINE AND SODIUM BENZOATE AFTER ORAL ADMINISTRATION TO RATS

BY

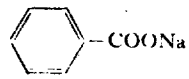
G. M. FANELLI AND S. L. HALLIDAY

(Received for publication 29-6-1962).

Among the uses proposed for chlortetracycline has been that of a food additive to retard bacterial spoilage. In view of this, it was deemed of interest to compare the relative toxicity of chlortetracycline with sodium benzoate (FIG. 1) since the latter is frequently cited as an example of a widely used food additive of low toxicity. However, sodium benzoate produces growth inhibition in rats when incorporated into the diet in high concentration (GRIFFITH, 1929; WHITE, 1941; KOWALEWSKI, 1960).



Chlortetracycline hydrochloride
M.W. 515



Sodium benzoate
M.W. 144

FIG. 1

Chemical structures of chlortetracycline and sodium benzoate.

METHODS

Thirty male and thirty female Sherman rats, 23 days old, weighing 40-50 g were assigned randomly to ten vertical groups each of six individual cages. Body weights were recorded and the animals observed grossly for any abnormalities. The rats were given a basal diet and water *ad libitum* for 3 days, after which they were fed their respective drug diets for 28 days.

The basal diet had the following composition:

ground yellow corn	62 pounds
soybean meal (44 %)	20 pounds
corn gluten meal (44 %)	5 pounds
fish meal	5 pounds
distillers solubles	2.5 pounds
alfalfa meal (20 %)	2 pounds
trace mineral concentrate[1]	2 pounds
steamed bone meal	1.5 pounds
iodized salt	0.5 pounds
choline chloride (25 %)	0.1 pounds
Vitamin A and D (dry)[2]	0.1 pounds
Vitamin mix[3]	0.1 pounds
Total	100.8 pounds

(1) Trace mineral concentrate (MIC 8009): (Limecrest Co., Newton, N. J.).

calcium	33-34.5 %
magnesium	2.6 %
manganese	0.20 %
iron	0.20 %
iodine	0.06 %
zinc	0.01 %
chloride	0.01 %
copper	0.022 %

(balance of percentage composition as carbonate)

(2) 10,000 units per gram of A.
2,000 units per gram of D_3.

(3) Vitamin mix.

ground yellow corn	43 grams
riboflavin	0.2 grams
niacinamide	0.4 grams
calciumpantothenate	1.8 grams
Vitamin B_{12} crystalline	0.5 milligrams

Food grade "once recrystallized" chlortetracycline hydrochloride was incorporated in the basal diet at concentrations of 5 % and 2 %. Similarly, sodium benzoate was added to a batch of the basal diet in concentrations of 5 % and 2 %.

The rats were weighed individually twice a week and were inspected daily for signs of toxicity. Food consumption for each group was recorded weekly and the drug intake as mg/kg rat/day was calculated using the average body weights for each group (TABLE I). Fisher's '*t*' test for small samples was used as a test for significant differences between body weights for the various groups.

RESULTS AND DISCUSSION

All animals receiving the 5 % sodium benzoate diet died during the first and second week following a period of hyperexcitability, urinary incontinence and convulsive seizures. A marked weight depression when compared with controls occurred in the rats receiving the 5 % chlortetracycline diet (FIGS. 2 and 3) but they appeared normal in all other respects.

Apart from a slight weight depression significant for male rats (TABLE II) the groups given 2 % sodium benzoate were similar to the controls.

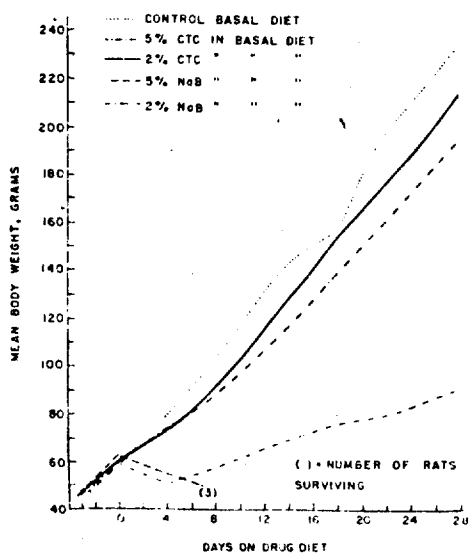


FIG. 2

Body weight of male rats fed chlortetracycline or sodium benzoate in diet for 28 days.

This is in general agreement with previous studies by GRIFFITH (1929). The groups maintained on the 2 % chlortetracycline diet were in excellent condition and final body weights were not significantly different from those of the controls but were significantly greater than the rats on 2 % sodium benzoate.

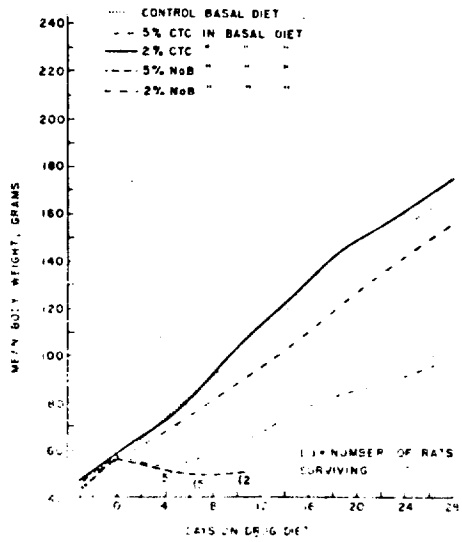


FIG. 3

Body weight of female rats fed chlortetracycline or sodium benzoate in diet for 28 days.

It will be seen from Table I that the average food consumption for the animals receiving 2 % chlortetracycline was comparable to that of the controls. Average drug intake of the rats eating the 2 % chlortetracycline diet was slightly more than that of the group ingesting the 2 % sodium benzoate and is accounted for by the decreased food consumption of the latter group after seven days.

Previous studies from these laboratories (DESSAU and SULLIVAN, 1961) have indicated that continual feeding of chlortetracycline to rats for two years did not produce manifestations of toxicity. Feeding experiments referred to previously (GRIFFITH, 1929; WHITE, 1941; KOWALEWSKI, 1960) were no longer than 6 weeks in duration.

On the basis of the data presented in Tables I and II, it is evident that chlortetracycline incorporated in the diet of rats is less toxic than comparable levels of sodium benzoate

TABLE I

Average Body Weight, Food Consumption, and Drug Intake for Rats Fed Chlortetracycline and Sodium Benzoate in the Diet for 28 Days

Drug Concentration in Diet	No. of Rats	Initial Weight-g	Days on Drug: 7			14			21			28		
			Average Body Weight-g	Food Consumption g/rat/day	Drug Intake mg/kg rat/day	Average Body Weight-g	Food Consumption g/rat/day	Drug Intake mg/kg rat/day	Average Body Weight-g	Food Consumption g/rat/day	Drug Intake mg/kg rat/day	Average Body Weight-g	Food Consumption g/rat/day	Drug Intake mg/kg rat/day
0	6 M	59	96	11		145	14	—	191	20	—	235	21	—
	6 F	57	86	10		119	14	—	146	16	—	168	17	—
5 % CTC	6 M	59	56	5	4165	69	9	6223	78	10	6066	91	12	6442
	6 F	58	56	5	4270	74	9	6321	86	11	6339	100	12	6106
2 % CTC	6 M	62	86	10	2261	128	16	2455	170	19	2217	214	23	2245
	6 F	60	86	10	2269	122	14	2380	151	17	2307	174	19	2172
5 % NaB	6 M[a]	63	50	6	5686	—	—	—	—	—	—	—	—	—
	6 F[b]	58	48	8	7780	—	—	—	—	—	—	—	—	—
2 % NaB	6 M	59	82	10	2357	116	10	2218	155	16	2089	195	20	2002
	6 F	56	77	9	2396	102	12	2299	130	14	2209	156	17	2171

[a] All dead after 11 days.
[b] All dead after 13 days.

TABLE II

Comparison of 28 Day Body Weights for the Various Groups

Group	Sex	P (1)
2 % Chlortetracycline vs. Controls	M	> 0.05
2 % Chlortetracycline vs. Controls	F	> 0.1
2 % Sodium Benzoate vs. Controls	M	< 0.001
2 % Sodium Benzoate vs. Controls	F	< 0.1
2 % Sodium Benzoate vs. 2 % Chlortetracycline	M	< 0.01
2 % Sodium Benzoate vs. 2 % Chlortetracycline	F	< 0.01

(1) Fisher's *t*'-test for small samples.

SUMMARY

The results of a study of the relative toxicity of chlortetracycline and sodium benzoate have been presented.

1. All animals eating the 5 % sodium benzoate diet died between the first and second week following a period of hyperexcitability, urinary incontinence and convulsive seizures.

2. Rats receiving the 5 % chlortetracycline diet had a marked reduction in final body weight but appeared as healthy as the controls in other respects.

3. The only effect noted in the rats consuming the 2 % sodium benzoate diet was a slight depression of body weight significant for males.

4. Rats maintained on the 2 % chlortetracycline diet maintained body weight comparable to the controls.

5. At the concentrations employed, chlortetracycline was less toxic when fed to rats than sodium benzoate.

ACKNOWLEDGMENT

The authors wish to thank Miss C. H. Hine and Mr. D. Hoyt for their assistance and Mrs. D. Lennon for aid in the preparation of the manuscript.

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Analyst 91: 15-22, 1966.

The Quantitative Determination of Benzoic Acid in Soft Drinks by Ion-Exchange Chromatography*

By M. A. FORD

(*Product Research Department, Beecham Food and Drink Division, Coleford, Gloucestershire*)

A quantitative method for the determination of benzoic acid in soft drinks and their associated bases and compounds is described. The method consists essentially in the isolation of benzoic acid from the sample by means of ion-exchange chromatography on De-Acidite FF anion-exchange resin, and the subsequent estimation of the isolated preservative by ultraviolet spectrophotometry. The separation of benzoic acid from other constituents is discussed and a simple three-point correction formula is proposed to correct for non-specific background absorbance. Recovery of benzoic acid was satisfactory when the method was applied to a wide range of ready-to-drink beverages, concentrated soft drinks, and fruit bases and compounds.

The analysis of foodstuffs for benzoic acid has been the subject of many papers. The first authoritative monograph was published by Monier-Williams in 1927,[1] and has long been accepted as the standard and recommended method of analysis[2] for this preservative.

Succeeding workers have omitted the initial "clean-up" stages recommended by Monier-Williams, and have simply extracted the benzoic acid with immiscible solvents.[3,4,5,6] Determination of the isolated acid has been made gravimetrically, after sublimation,[1,7] titrimetrically with a standard base[4] and colorimetrically.[8,9]

Ultraviolet spectrophotometry was preferred by some workers,[5,6,10] since it was more specific than simple titrimetric methods. However, this technique is vitiated when saccharin is present and diethyl ether has been used as the extractant.

Recently, newer analytical techniques have been applied to the problem, and papers have appeared in which column chromatography,[11] paper chromatography and electrophoresis[12,13,14,15] and gas chromatography[16,17] have been used. Since immiscible solvents tend to extract interfering materials and both Monier-Williams's method and the more recent chromatographic techniques did not lend themselves to the type of rapid routine estimation envisaged, a suitable alternative was sought.

In 1956, Davies and Owen[18] reported the separation of benzoic acid from acetic and phenylacetic acids on a strongly basic anion-exchange resin, and 0·1 N hydrochloric acid in aqueous dioxan as the eluting solution. Preliminary experiments with a similar type of resin, De-Acidite FF, yielded promising results, and therefore a more detailed investigation was made with this resin.

Experimental

Choice of ion-exchange resin—

Two ion-exchange resins were examined during the investigation, namely, De-Acidite FF and Dowex 1 — X10. The former resin was preferred because of the slow flow-rate through the Dowex resin, apparently due to the high proportion of fine particles present, and because of an artifact that was eluted from this resin, causing an error in the spectrophotometric determination of benzoic acid. As Davies and Owen did not specify the degree of cross-linking in the resin that they used, it was necessary to experiment with the 3 grades that were available, *viz.*, 2 to 3 per cent., 3 to 5 per cent. and 7 to 9 per cent. of cross-linking. It was found that none of these variants affected the position at which benzoic acid was eluted, although cross-linking has a more pronounced effect on larger molecules. It was therefore decided to adopt De-Acidite FF resin with 3 to 5 per cent. cross-linking and of 100 to 200 mesh, the latter being an acceptable compromise yielding maximum flow-rate for minimum tailing of the eluted peaks.

Early experiments were conducted in the conventional manner, in which the resin was converted to the free-base form and the column developed with solutions of chloride ions either as hydrochloric acid or as sodium chloride. It was found that the use of the resin in the free-base form necessitated the use of reagents free from carbon dioxide because of

* Presented at the meeting of the Society on Wednesday, March 31st, 1965.

its rapid absorption by strongly basic resins to yield the carbonate form of the resin. In addition to proving inconvenient, use of solutions of sodium chloride for elution led to the formation of a precipitate of sodium carbonate if the effluent from the column was allowed to stand for a short period.

Strongly basic ion-exchange resins have the following ionic affinities in dilute solution[19,20]—

Sulphate > citrate > bisulphite > chloride > carbonate > hydroxide.

Exchange will only occur if the ion on the resin has a lower affinity for the resin than the ion in solution. If the resin is used in the free-base form, *i.e.*, the hydroxide form, all anions will be exchanged. If however, the resin is used in the chloride form, then all anions except the carbonate and the hydroxide ions will be exchanged. The use of the resin in the chloride form also obviates the need for reagents free from carbon dioxide and the lengthy regeneration procedure that was previously necessary.

CHOICE OF ELUTING SOLUTION—

The preliminary experiments were carried out with Davies and Owen's eluting solution, *i.e.*, 0·1 N hydrochloric acid - 35 per cent. dioxan. This proved unsatisfactory for two reasons however, first dioxan is unsuitable as a spectrophotometric solvent as it absorbs light strongly at all wavelengths below 260 nm, and secondly, the use of 0·1 N hydrochloric acid caused marked disturbance of the resin bed owing to the formation of pockets of vapour.

Reduction in the strength of the hydrochloric acid solution and elimination of the dioxan overcame these objections and recoveries of benzoic acid were satisfactory. However, when elution curves of the major constituents of soft drinks were prepared, it was found that, while most of the compounds were separated from benzoic acid, citric acid and malic acid failed to separate completely. Although neither of these acids possesses any appreciable absorption in the ultraviolet region, both are potential sources of error and should be excluded if possible.

Early work had shown that sodium chloride solutions could also be used to develop the column. Satisfactory separation could be achieved with a 0·1 M solution of sodium chloride, but was accompanied by considerable tailing of the benzoic acid peak and a reduction in the amount of benzoic acid recovered. It was thought that the low recoveries could be attributed to molecular adsorption effects, since the inclusion of an aromatic substituent in a molecule causes an acid to be retained more strongly than its aliphatic analogue. Davies and Owen found that the use of mixed solvents considerably reduced this effect, and as ethanol is a more suitable spectrophotometric solvent than most, the effect of adding this solvent to the sodium chloride solution was investigated.

In addition to the improvement in the average recovery of benzoic acid, shown in Table I, the tailing of the benzoic acid peak was reduced.

TABLE I

THE EFFECT OF ETHANOL CONCENTRATION ON THE RECOVERY OF BENZOIC ACID

Ethanol added, per cent.	0	10	20	40	50	75
Benzoic acid recovered, per cent.	91·3	92·8	96·1	95·4	99·0	99·4

It was also found that the position at which benzoic acid emerged was altered, the acid appearing earlier with progressively increasing concentrations of ethanol. This is because solubility also has an effect and those acids that are more soluble in alcohol will emerge earlier, while the emergence of those that are more soluble in water will be retarded. It was found necessary therefore to adjust the ethanol concentration of the sodium chloride solution not only to yield satisfactory recoveries of benzoic acid, but also to achieve optimum separation from interfering compounds, and while a concentration of 50 per cent. ethanol gave satisfactory recoveries of benzoic acid, a concentration of 75 per cent. ethanol was necessary to effect complete separation from the other acids.

CHOICE OF CONDITIONS FOR THE COLUMN—

If amounts of citric acid of the same order as those found in soft drinks were added to the ion-exchange column, the breakthrough capacity of the resin was exceeded and citrate ions appeared in the early fractions of the effluent. In order to separate the relatively large amounts of citric acid from benzoic acid, a large column would be necessary with the resultant

decrease in flow-rate and increased time of analysis. This could be avoided by the prior removal of the bulk of the citrate ions, by making use of the fact that the calcium salts of citric and malic acids are insoluble in alcoholic solution while that of benzoic acid is soluble. Addition of calcium carbonate to a solution containing both citric acid and benzoic acid, and subsequent addition of ethanol to yield a final ethanolic concentration of 50 per cent., resulted in the precipitation of approximately 80 to 90 per cent. of the interfering acids, while an average of only 2·4 per cent. of the benzoic acid was lost. This loss is due to the cumulative errors caused by molecular-adsorption effects, sorption on the calcium citrate precipitate and manipulative losses. It is therefore recommended that the calibration graph should be prepared under the same conditions.

By using the calcium precipitation technique, the size of the column could be reduced, and a column formed by transferring 5 g of moist resin to a chromatography column 1 cm in internal diameter gave satisfactory results. Under these conditions, with a constant-head reservoir of the type described by the author,[21] a flow-rate of 1 ml per minute was readily attainable.

The exchange capacity of De-Acidite FF is largely independent of pH, and although the pH of the sample is raised from about 3 to between 7 and 8 by the calcium precipitation technique, this has no effect on the exchange capacity of the resin.

SPECTROPHOTOMETRY—

The benzoic acid is eluted from the column in the form of its sodium salt, and it was decided to acidify the solutions of sodium benzoate in order to utilise the bathochromic displacement of the position of the peak into the region in which the background absorbance is linear. The difference in the positions of $\lambda_{max.}$ of sodium benzoate solutions in neutral and in acid solutions is shown in Fig.1. The use of acid concentrations from 0·2 N to 2 N for spectrophotometry had little effect on the spectrum of benzoic acid, but an increase in the hydrochloric acid concentration to 5 N caused a further shift in the position of $\lambda_{max.}$ with a concomitant decrease in intensity. A final concentration of 1 N was therefore used in the method.

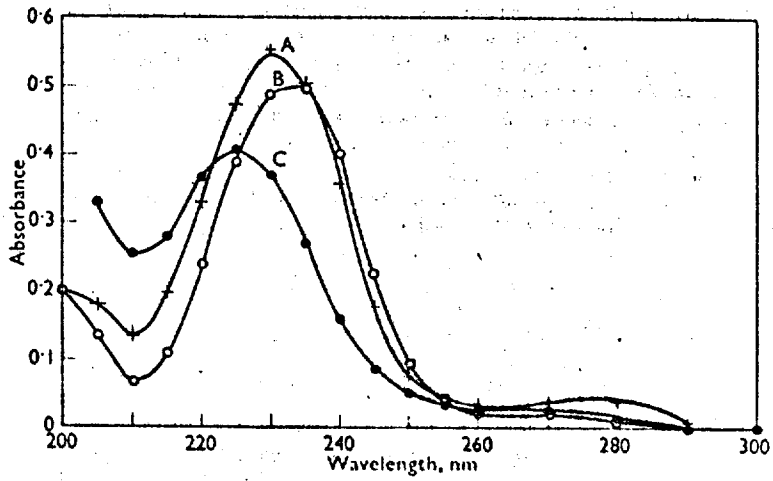

Fig. 1. Ultraviolet spectra of solutions of sodium benzoate in neutral and acid media: curve A, acidified to N with hydrochloric acid; curve B, acidified to 5N with hydrochloric acid; curve C, neutral solution

The presence of variable background absorbance gave insufficiently precise results. Since the irrelevant absorbance was found to be linear over that portion of the spectrum in which the benzoic acid peak occurred, it was found possible to use a correction of the type proposed by Morton and Stubbs,[22] and expanded by Allen.[23]

The general equation is—

$$E_{max.}^{corr.} = E_{max.} - [K(E_1 - E_2) + E_2] \quad \ldots \quad \ldots \quad \ldots \quad (1)$$

$$K = \frac{\lambda_2 - \lambda_{max.}}{\lambda_2 - \lambda_1}$$

For the proposed method the wavelengths of 220 and 245 nm were chosen, together with $\lambda_{max.}$ of 230 nm, since this is the range over which the background absorbance is virtually linear.

Therefore—

$$K = \frac{245 - 230}{245 - 220} = 0{\cdot}6$$

and substituting this value in equation (1) and re-arranging, it becomes

$$E_{230}^{corr.} = E_{230} - (0{\cdot}6\ E_{220} + 0{\cdot}4\ E_{245}) \quad \ldots \quad \ldots \quad \ldots \quad (2)$$

where E_{220}, E_{230} and E_{245} represent the observed absorbances at these wavelengths.

Because of the necessity for the background absorbance to be linear, it is recommended that the "blank" spectrum should be determined initially, wherever practicable, although the irrelevant absorbance of a wide range of fruit products is in fact linear over this narrow range.

During a study of product blanks it was found that certain of them exhibited selective absorbance at 245 nm, found to be due to ascorbic acid, part of which is eluted in the same fraction as benzoic acid. When the ion-exchange resin had been used in the free-base form, the ascorbic acid was broken down on the column and could not be detected in the effluent. However, with the use of the resin in the chloride form, this no longer occurred and, since ascorbic acid has a high molecular extinction coefficient ($\log \epsilon = 4{\cdot}03$), its potential interference was great. Although the correction formula given above would eliminate most of the interference from the ascorbic acid, it would be more satisfactory if this interference could be eliminated entirely. If ascorbic acid is oxidised to dehydroascorbic acid, the absorbance at 245 nm disappears. This oxidation is readily accomplished by the addition of a small amount of copper ions to the solution, to catalyse the aerial oxidation of ascorbic acid. With this technique, the interference from this source was completely eliminated and the recovery of benzoic acid from products containing ascorbic acid improved.

METHOD

APPARATUS—

Chromatography columns—All-glass chromatography columns, 1 cm in internal diameter, 30 cm in length, provided with a sintered-glass disc and a stopcock at the lower end, and a B14 standard ground-glass joint at the upper end.

Constant-head reservoir—Details of the construction and use have been described by Ford.[21] The reservoir should have a capacity of about 250 ml and be provided with a B14 standard ground-glass joint.

Sintered-glass discs—1 cm in diameter, porosity 0.

For a diagram of the complete apparatus, see Fig. 2.

Spectrophotometer—A Unicam SP500 spectrophotometer was used throughout this investigation.

REAGENTS—

Analytical-grade reagents should be used wherever practicable.

Ion-exchange resins—Permutit De-Acidite FF ion-exchange resin*, chromatographic grade, chloride form, 100 to 200 mesh, 3 to 5 per cent. average cross-linking. Prepare the resin by transferring it to a large sintered-glass funnel and washing it with distilled water until any foreign matter, which may be present, is removed. Remove the bulk of the water by suction and store the resin in a moist state.

Calcium carbonate.

Ethanol, absolute, B.P. quality.

Ethanol, 75 per cent. v/v.

Eluting solution—Dissolve 5·85 g of sodium chloride in 250 ml of water, and when dissolved dilute to 1 litre with absolute alcohol and mix.

Hydrochloric acid, 5 N.

Cupric sulphate, 0·2 *per cent. w/v.*

Standard benzoic acid solution—Dissolve 100 mg of benzoic acid in water and dilute the solution to 100 ml with water.

* The Permutit Company Ltd. now produce De-acidite FF resin in an isoporous form in addition to the previously available macroporous type that was used for the investigation. The isoporous form of the resin is now denoted by the letters 'I.P.' after the code number, and it is necessary to specify De-acidite FF anion exchange resin 'old type' when ordering this material for use with this method.

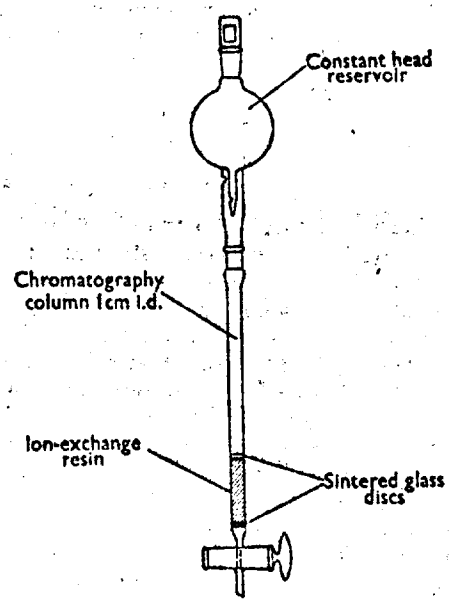

Fig. 2. Chromatographic apparatus

PREPARATION OF ION-EXCHANGE COLUMN—

Transfer 5 g of the moist resin to the chromatography column, and allow to settle. Then back-flush the resin bed with distilled water, stirring the resin with a glass rod to disperse any air bubbles that may be present. Again allow the resin bed to settle and pass 50 ml of distilled water through the resin. When the level of the water has almost reached the resin, place a sintered-glass disc on the top of the resin bed to prevent disturbance of the column during additions. Finally allow the level of the water to drain to the top of the resin bed, and the column is then ready for use. The resin must be kept moist at all times, and a fresh column of resin must be used for each test.

PREPARATION OF CALIBRATION GRAPH

Transfer 0·5, 1·0, 2·0, 3·0, 4·0 and 5·0-ml aliquots of the standard benzoic acid solution (1·0 mg per ml), to 15-ml graduated centrifuge tubes containing 0·2 g calcium carbonate. Add water as necessary to adjust the volume of each to 5 ml, mix, then dilute to 9·6 to 9·8 ml with ethanol and again mix thoroughly with a small stirring rod. Wash the stirring rod and the sides of the tube with a small amount of 75 per cent. ethanol and adjust the final volume to about 10 ml. Spin the sample in a centrifuge at 3000 r.p.m. for 2 to 3 minutes.

Place a 25-ml measuring cylinder under the column and transfer the supernatant liquid from the centrifuge tube to the ion-exchange column prepared as detailed above, by means of a dropping pipette. Wash the precipitate successively with two 4-ml portions of 75 per cent. ethanol. Wash the stirring rod and sides of the tube with the diluted ethanol as before; ensure that the final volume is about 5 ml on each occasion. Transfer the supernatant liquid from each washing, obtained by centrifuging the suspensions, to the column. When the final wash solution has drained to the level of the resin, elute the benzoic acid by adding 75 ml of the eluting solution to the column, using the constant-head reservoir, and at a flow-rate of about 1 ml per minute. Continue until 25 ml of the effluent are collected, then replace the cylinder with a 50-ml graduated flask containing 15 ml of eluting solution, and continue collecting until the graduation is reached; in this way the succeeding 35 ml of effluent are collected, and retained for analysis.

Mix this portion of the eluate; transfer a 5-ml aliquot to a 50-ml graduated flask, and add 10 ml of 5 N hydrochloric acid and 1 ml of 0·2 per cent. cupric sulphate solution. Mix, and allow it to stand for 10 minutes, then dilute to 50 ml with water; simultaneously, prepare a blank by mixing 5 ml of eluting solution, 10 ml of 5 N hydrochloric acid, 1 ml of 0·2 per cent cupric sulphate solution and dilute the whole to 50 ml with water. Measure the absorbance

of the solutions at 220, 230 and 245 nm, in 1-cm fused-silica cells and with the blank as a reference solution. Substitute the observed absorbances in equation (2), *i.e.*,

$$E_{230}^{corr} = E_{230} - (0{\cdot}6\ E_{220} + 0{\cdot}4\ E_{245})$$

From the corrected readings construct a calibration graph relating corrected absorbance at 230 nm to concentration of benzoic acid in μg per 50 ml of solution. Calibration graphs prepared as described above have been found to be remarkably constant and only need checking at infrequent intervals.

PROCEDURE—

For soft drinks containing 100 to 800 p.p.m. of benzoic acid, transfer a 5-g aliquot of a well mixed sample to a 15-ml graduated centrifuge tube containing 0·2 g calcium carbonate and continue as described above, under "Preparation of Calibration Graph."

For bases and compounds containing 800 to 2000 p.p.m. of benzoic acid, dilute 20 g to 40 g of a well mixed sample to 100 g with water, and mix thoroughly. Then transfer a 5-g aliquot of the diluted base or compound to a 15-ml graduated centrifuge tube containing 0·2 g of calcium carbonate and continue as described above, under "Preparation of Calibration Graph."

Similarly, correct the absorbance of the sample solutions as described above by using equation (2), and by reference to the calibration graph, determine the concentration of benzoic acid in the measured solution. Designate this concentration A μg per 50 ml of solution.

CALCULATION—

For soft drinks containing 100 to 800 p.p.m. of benzoic acid,

Benzoic acid content of sample in parts per million

$$= \frac{A \times \text{Volume of eluate} \times 10^6}{\text{Aliquot of eluate} \times \text{Weight of sample} \times 10^6}$$

$$= \frac{A \times 50 \times 10^6}{5 \times \text{Weight of sample} \times 10^6}$$

$$= \frac{10\ A}{\text{Weight of sample}}$$

For bases and compounds containing 800 to 2000 p.p.m. of benzoic acid,

Benzoic acid content of sample in parts per million

$$= \frac{A \times \text{Volume of eluate} \times \text{Weight of diluted base} \times 10^6}{\text{Aliquot of eluate} \times \text{Weight of aliquot of diluted base} \times \text{Weight of base} \times 10^6}$$

$$= \frac{A \times 50 \times \text{Weight of diluted base} \times 10^6}{5 \times \text{Weight of aliquot of diluted base} \times \text{Weight of base} \times 10^6}$$

$$= \frac{10\ A \times \text{Weight of diluted base}}{\text{Weight of aliquot of diluted base} \times \text{Weight of base}}$$

RESULTS AND DISCUSSION

In order to test the accuracy and precision of the proposed method, recovery experiments were carried out on 12 differing types of fruit juice products and the results of those analyses are summarised in Table II.

For the method to be viable it is necessary for most of the principal constituents of fruit juice products to be separated from the benzoic acid, because the background absorbance must be linear. A study of the ultraviolet absorption spectra of citric acid, malic acid, lactic acid, ascorbic acid, saccharin, cyclohexylsulphamic acid (cyclamate), sugars, quinine, naringin, artificial and natural colours and inorganic ions, revealed that all except cyclohexylsulphamic acid, the sugars and inorganic ions possessed appreciable absorbance in the region under examination. Reference to Table III shows that the substances that would cause the most serious interference are ascorbic acid, saccharin, quinine and naringin, while citric acid, malic acid and, to a lesser extent, lactic acid would cause slight disproportionate errors at 220 and 230 nm.

TABLE II

THE RECOVERY OF BENZOIC ACID ADDED TO SOFT DRINKS, FRUIT BASES AND COMPOUNDS

Product	Benzoic acid added, p.p.m.	Benzoic acid recovered, p.p.m.	Number of determinations	Average recovery, per cent.	Standard deviation, p.p.m.
Orange base	1790	1779–1807	12	99·7	9·7
Orange drink	558	548–562	12	99·6	4·0
Orange drink	154	141–160	6	100	4·0
Carbonated orange drink ..	100	90–96	12	93	1·8
Lemon base	1799	1768–1817	6	99·6	19·8
Lemon drink	441	428–432	6	97·6	2·1
Bitter lemon drink	425	405–414	6	96·3	3·5
Lemon barley drink	423	405–413	6	96·9	3·0
Carbonated bitter lemon drink ..	100	90–94	6	92	2·0
Glucose drink	216	210–214	6	98·5	1·5
Apple compound	601	593–600	6	99·1	2·5
Blackcurrant juice drink	160	150–152	6	94·3	0·8

TABLE III

MOLECULAR EXTINCTION COEFFICIENTS OF THE PRINCIPAL CONSTITUENTS OF SOFT DRINKS

Substance	Molecular extinction coefficient	Wavelength, nm
Benzoic acid	11350	230
Citric acid	200	209
Malic acid	160	209
Lactic acid	70	209
Ascorbic acid	10640	245
Saccharin	29670	205
Quinine	31540	208
	29920	250
Naringin	26820	212
	16350	286

The elution diagrams of these principal constituents were determined and are as shown in Fig. 3. Most of the compounds were separated satisfactorily with the exception of lactic acid and naringin, while malic acid and ascorbic acid were incompletely separated.

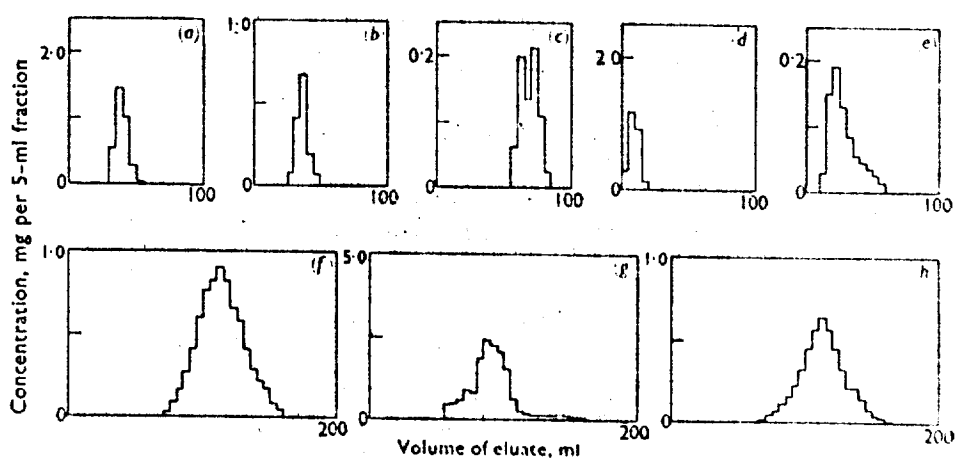

Fig. 3. Elution curves of the principal constituents of soft drinks: (*a*) benzoic acid; (*b*) lactic acid; (*c*) ascorbic acid; (*d*) quinine; (*e*) naringin; (*f*) citric acid; (*g*) malic acid; (*h*) saccharin

The interference from ascorbic acid is overcome by oxidising this compound to dehydroascorbic acid, and since both malic acid and lactic acid have low molecular extinction coefficients, concentrations of these two acids several times higher than those encountered in practice can be tolerated without causing interference. Similarly, in the case of naringin,

those concentrations which are normally present in grapefruit drinks do not cause any error in the spectrophotometric estimation of benzoic acid, although it is possible that samples containing an inordinately high concentration of this flavonoid may be subject to erratic results.

Conclusions

It has been shown that the use of ion-exchange chromatography with mixed solvents, together with the use of ultraviolet spectrophotometry provides a rapid and precise method of analysis for benzoic acid, the time required being approximately 2 to 2½ hours. The accuracy and precision that is obtainable is considered satisfactory for the routine type of analysis required, and the basic method can be used for the estimation of benzoic acid in soft drinks and their associated products in the range of 100 to 2000 p.p.m.

I thank the Directors of Beecham Food and Drink Division for permission to publish this paper, and also my colleagues, Mr. A. G. Wells for his advice and encouragement, and Mr. D. C. Cook for his valuable technical assistance.

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Received *June 30th*, 1965

PRESERVATIVES AND ARTIFICIAL SWEETENERS

Rapid Screening Procedure for Determination of Benzoic Acid and Sorbic Acid in Fruit Beverages

By W. M. GANTENBEIN and A. B. KARASZ (New York State Food Laboratory, Department of Agriculture and Markets, 1220 Washington Ave., Albany, N.Y. 12226)

A rapid and accurate method, requiring one extraction and UV analysis, has been developed for simultaneous detection of benzoic and sorbic acids in fruit beverages. Quantitative measurements of benzoic and sorbic acids are based on the strong absorbances at 225 and 250 mμ, respectively. Recoveries of benzoic and sorbic acids from orange juice and apple juice at levels normally present were adequate.

One of the routine tasks of this laboratory is the detection of undeclared preservatives in fruit beverages; the most commonly suspected preservatives are benzoic and sorbic acids. Since a large number of samples must be examined to find the relatively few violations, there is a need for a rapid screening test.

The official AOAC spectrophotometric method, 27.008, for benzoic acid in beverages requires 15 consecutive extractions and is not suitable for processing a large number of samples. In the official method the benzoic acid is estimated from the absorption of the extract at 272 mμ where benzoic acid has an absorption maximum. To obtain a suitably high absorption at the commonly occurring benzoic acid concentrations, a 10 ml beverage sample is analyzed; however, the extract from this size sample contains enough of a UV absorbing material so that correction is required.

The proposed rapid screening method for benzoic acid is based on the fact that, in addition to the absorption maximum at 272 mμ and the two adjacent lesser ones at 282 and 265 mμ (1), benzoic acid also has a maximum at 225 mμ (2); the absorptivity at this wavelength is about ten times greater than that at 272 mμ, as shown in Fig. 1. Consequently, measuring the absorption at 225 mμ permits the use of a much smaller sample: 1.0 ml is sufficient. This small volume can be dissolved in ethyl ether, which then can be dried by adding petroleum ether and anhydrous sodium sulfate. Purification by extraction is not needed, as the concentration of UV-absorbing materials in the small volume of fruit beverage taken for analysis is quite low.

This rapid test will also reveal the presence of sorbic acid, which absorbs strongly at 250 mμ (Fig. 2). Sorbic acid may be quantitatively measured by its absorption at 250 mμ.

We have not encountered beverage samples containing both benzoic and sorbic acids. Such a combination would have absorption maxima at 250, 225, and 207 mμ; *see* Fig. 3. A rough estimate of the amount of sorbic acid may still be made, since the absorption of benzoic acid at 250 mμ is at a minimum. The quantitation of benzoic acid in such a mixture

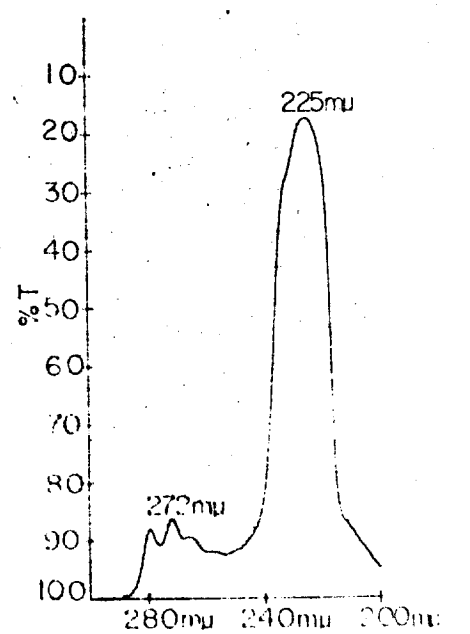

FIG. 1—UV spectrum of 0.10% benzoic acid in orange juice.

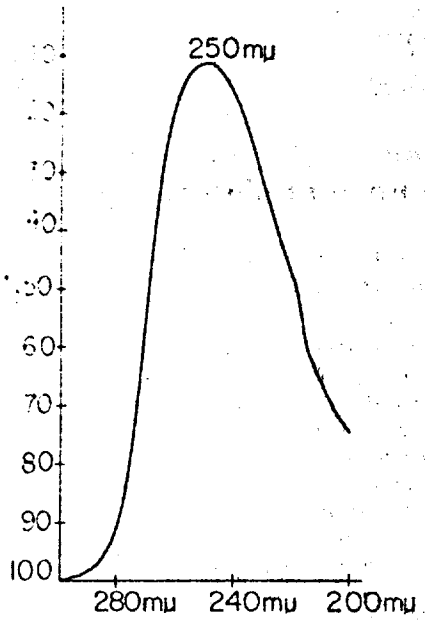

FIG. 2—UV spectrum of 0.04% sorbic acid in orange juice.

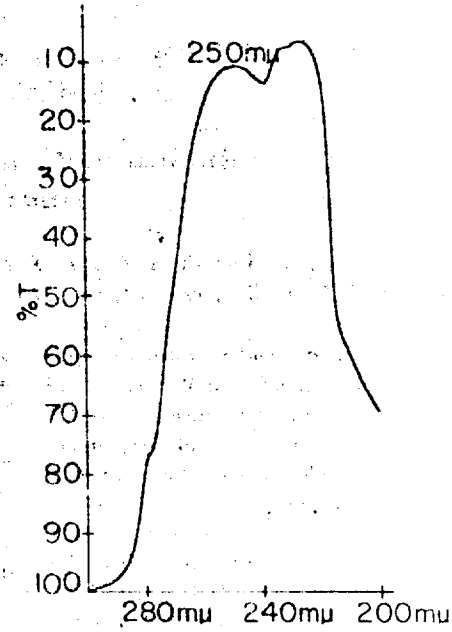

FIG. 3—UV spectrum of 0.10% benzoic acid and 0.04% sorbic acid in orange juice.

requires separation of the two acids by thin layer or gas chromatography.

In the application of this method we have not encountered any interferences. However, fumaric acid has been approved as an acidulant in beverages and it has an absorption maximum at 225 mμ. Fumaric acid can be distinguished from benzoic acid, since it does not exhibit either a triple peak at 282, 272, and 265 mμ or a shoulder at 233 mμ. We have not found fumaric acid in any of the fruit beverages we have examined.

In non-fruit beverages many UV-absorbing materials, such as vanillin and other flavors, may be expected, so the method has to be applied to such samples with caution.

METHOD

Apparatus and Reagents

(a) *Spectrophotometer.* Beckman DB, or equivalent, with recorder.

(b) *Hydrochloric acid.* 1 + 1.

(c) *Benzoic acid solutions.*—(*1*) *Stock solution:* Dissolve 0.5900 g sodium benzoate in water and dilute to 100 ml. (*2*) *Standard solutions:* Dilute 4.0, 8.0, 12.0, 16.0, and 20.0 ml stock solution to 100 ml with water.

(d) *Sorbic acid solutions.*—(*1*) *Stock solution:* Dissolve 0.6700 g potassium sorbate in water and dilute to 200 ml. (*2*) *Standard solutions:* Dilute 2.0, 4.0, 8.0, 12.0, and 16.0 ml stock solution to 100 ml.

Preparation of Standard Curve

Pipet 1.0 ml of each standard solution into glass-stoppered Erlenmeyer flask and acidify with 0.5 ml HCl (1 + 1). Add 40 ml ethyl ether, 40 ml petroleum ether, and ca 5 g anhydrous sodium sulfate separately and shake after each addition. Filter into 100 ml volumetric flask, and wash Erlenmeyer flask and filter three times with ether; dilute to volume with ether. Prepare blank by treating 1.0 ml water in same manner. Record absorbance of the standard solutions against blank, using 1 cm cells.

For benzoic acid, plot concentration against absorbance at 225 mμ. For sorbic acid, plot concentration against absorbance at 250 mμ. These plots are linear for benzoic acid up to 1.0 mg and for sorbic acid up to 0.4 mg in 100 ml ether solution, as shown in Fig. 4, and obey the Beer-Lambert law.

Determination

Analyze 1.0 ml beverage as described above for the standard. From the absorbance of sample, find in standard plot corresponding concentration of preservative in 1.0 ml sample. Multiply

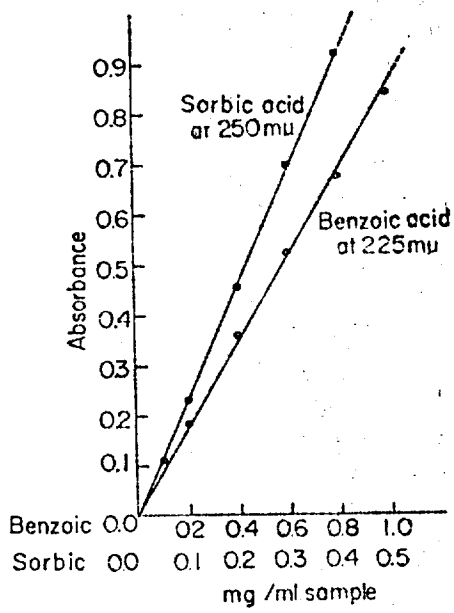

FIG. 4—Calibration curves for benzoic acid and sorbic acid.

this value by 100 and report result as mg preservative/100 ml beverage.

If concentration of preservative exceeds that of standard plot, dilute beverage with water and repeat determination.

Results and Discussion

The Food Additives regulations (3) permit the use, with declaration, of 0.1% benzoic and sorbic acids. The actual amounts used in commercial practice generally range between 0.04 and 0.1%. Sorbic acid usually is found near or below the lower value and benzoic acid closer to 0.1%.

We spiked authentic orange and apple juices prepared in the laboratory with sorbic and benzoic acids at the levels indicated by the above considerations. The highest spike for benzoic acid was 0.1% and for sorbic acid 0.04%. The sorbic acid range was kept lower partly because of its use as a preservative at a lower concentration and partly because a greater concentration yields excessive light absorption and the solution must be diluted as explained in the *Method*.

The results (Table 1) indicate that at the preservative concentrations generally used in juices the recoveries are reasonably good. At the lowest concentrations examined the recoveries were high; however, these are below the effective preservative levels and are not likely to be encountered.

Table 1. Recovery of benzoic and sorbic acids from fruit juices

Benzoic Acid, mg/ml			Sorbic Acid, mg/ml		
Added	Found	% Rec.	Added	Found	% Rec.
		Orange Juice			
0.200	0.255	127.5	0.040	0.057	142.5
0.400	0.395	98.8	0.100	0.120	120.0
0.600	0.540	90.0	0.200	0.210	105.0
0.800	0.730	91.2	0.300	0.300	100.0
1.00	0.835	83.5	0.400	0.395	98.8
0.200	0.240	120.0	0.200	0.222	110.0
0.600	0.547	91.1	0.300	0.310	103.0
1.00	0.820	82.0	0.400	0.418	104.5
		Apple Juice			
0.200	0.220	110.0	0.050	0.060	120.0
0.300	0.280	93.2	0.100	0.112	112.0
0.400	0.360	90.0	0.150	0.142	94.6
0.500	0.480	96.0	0.200	0.205	102.5
0.600	0.600	100.0	0.250	0.250	100.0
0.750	0.710	94.6	0.300	0.312	103.9
0.800	0.780	97.5	0.350	0.345	98.5
1.000	0.950	95.0	0.400	0.417	103.0

The high values found at the low preservative concentration are undoubtedly due to the background absorption of the juice which assumes greater significance at this level although, under the conditions of this test, the UV absorption of fruit juices is rather small, as it may be seen from typical absorption curves obtained for an authentic orange juice (Fig. 5, top) and an authentic apple juice (Fig. 5, bottom).

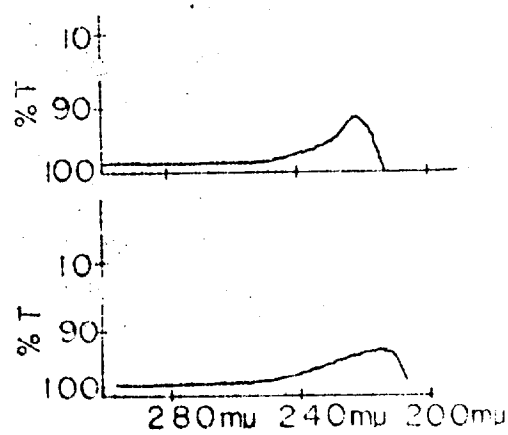

FIG. 5—UV absorption spectra of authentic orange juice (top) and authentic apple juice (bottom).

The absorption curves in Figs. 1–3 were obtained by spiking authentic orange juice with the preservatives indicated. These curves are characteristic and easily recognized. The benzoic acid peak at 225 mμ is not symmetrical and shows a noticeable shoulder at 235 mμ.

Summary

The method described is a rapid screening method for analyzing a large number of samples. It was primarily developed for beverages where undisclosed preservatives have been used. Compared to the official method, which requires 15 extractions and is time-consuming, this method is rapid and accurate for levels of preservatives normally present. In addition it is capable of detecting both sorbic and benzoic acids.

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Received October 25, 1968.

Physiological Effects of Benzoic Acid and Sodium Benzoate
(Verlag von Heinrich Staadt, Wiesbaden)

V. Gerlach

VII. Summary of the Results (Pages 90-92)

1. Benzoic acid and sodium benzoate had a preservative action in the amount of 1 part per 1000 for chopped meat, and in the same concentration prevented mold formation on margarine for 52 days.

2. Cold-blooded and warm-blood (frogs and rabbits) tolerate considerable amounts of benzoic acid or sodium benzoate by various routes of administration. This is also the case when the benzoates are administered for a longer period of time. The subcutaneous injection of 1g of sodium benzoate per kg body weight for 12 days resulted in no weight loss in test rabbits.

Large doses of approximately 1g of sodium benzoate per kg body weight temporarily reduce the body temperature of rabbits. The influence on the respiration and pulse was not evident. 2.5g per kg body weight of sodium benzoate (and more) were lethal.

5g benzoic acid or 7g sodium benzoate fed in 500 ml milk results in no detectable effect on respiration, pulse frequency, or body temperature of an average weight dog.

In a self-experiment, the intake of 10g benzoic acid within $3\frac{1}{2}$ hours resulted in absolutely no effect on body temperature, pulse, respiration, digestion, or any other untoward effects.

0.5g or 1.0g benzoic acid administered daily for 44 consecutive days did not show any visible effects in any experimental individuals, that is, body weight remained stable.

The effect on respiration, pulse, and body temperature after intake of 1g or 2g of benzoic acid or sodium benzoate showed no detectable effect after close observation.

3. Benzoic acid and sodium benzoate affects RBC as any acid or salt would.

Injection of 4 ml of a 5% solution of sodium benzoic had no effect on the frequency of heartbeat of frogs; even the injection of 1 ml of 20% sodium benzoic solution showed no effects after 1 hour.

Intravenous injection of sodium benzoate has a weaker effect on the suspended frog heart than an equally concentrated sodium-benzoic solution. The same effect is noted for cat hearts.

The average blood pressure of rabbits was only affected to a very minor degree and only transiently increased.

4. The effectiveness of digestive enzymes, gastric juice trypsin, and diastatic enzyme component was not affected in these experiments by the addition of sodium benzoic and benzoic acid. There was no effect from 3 or 5, sodium benzoic or benzoic acid on the amount, acid content, and digestive strength of the digestive juice in a Pavlovian-conditioned dog.

0.5g or 1.0g benzoic acid or sodium benzoate was administered in a test breakfast to the author, Gerlach. This dosage of benzoic acid or sodium benzoate had no effect on free salt acids,

total acidity, or digestive strength of the gastric juice which was siphoned out after one hour.

In human metabolic experiments, it was determined that daily intake of 1g benzoic acid or 1.5g sodium benzoate daily for 6 days consecutively had no effect on serum albumin or on the utilization of nitrates and food fats.

82 daily doses over a period of 86 days or 88 daily doses over 92 days of 1g each of benzoic acid had no recognizable unfavorable effects on body temperature, etc. At the end of the experiment, benzoic acid, whether mixed with food or occurring naturally in vegetables, was transformed completely into hippuric acid.

5. The literature reports that large doses of benzoic acid or sodium benzoate have been administered to individuals for both therapeutic and experimental purposes without resulting in harmful effects on health (negative side effects). Amounts of appr. 10g daily cannot be considered large in this sense.

6. Neither the experience in medical practice recorded in the literature nor the numerous experiments cited give justification for the assertion that benzoic acid or sodium benzoate can cause any kind of harm in the small amounts encountered in food preservatives. The attempt to ban benzoic acid or benzoates as preservatives would therefore not be justified by the voluminous scientific data available.

Proc. Soc. Exp. Biol. Med. 26(5): 354-5, 1929.

4300

Growth of Rats on Diets Containing Sodium Benzoate.

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The increase in weight of young male white rats during a 40 day experimental period on diets containing sodium benzoate has been determined. The food consumption was regulated so that all of the rats received equal quantities of food of equivalent calorific value. The increase in weight of rats receiving the basal diet plus 1.5, 2.0 or 2.5% sodium benzoate was practically the same as that of rats receiving the basal diet. On the diet containing 3% sodium benzoate one-third of the rats died and the growth of the remainder

[1] Somogyi, *J. Biol. Chem.*, 1928, xxviii, 9.
[2] Somogyi, *J. Biol. Chem.*, 1927, lxxv, 33.

was distinctly less than that of the control rats even though the food consumption was the same. The addition of glycine or of gelatin to the diet containing 3% sodium benzoate resulted in growth equal to that of rats on the basal diet. These results, based upon the consumption of equal amounts of food of the same calorific value, are in agreement with our earlier experiments in which similar diets were fed *ad libitum*.

The Toxicity of Benzoic Acid for White Rats*

By George P. Hager,† C. W. Chapman and E. B. Starkey

'nzoic acid is widely used in the preser- 'on of food products (1) and pharmaceuti- 'l preparations (2), especially those with acid reaction. Furthermore, because of antiseptic action, it is used in ointments ! dusting powders for the treatment of n infections, in a 1% solution as a mouth h (3), in a 0.5% solution for the treat- t of chronic suppurating wounds (4) ! in preparations for the treatment of reulosis, asthma and rheumatism (1).

n view of these many varied uses of ben- acid, much interest attaches to its toxic- It has been found that a rather broad margin of safety attends its use in these instances—0.5 Gm. daily produced no demonstrable effects in healthy persons and even 4 Gm. was not injurious (5, 6). Amounts far in excess of the quantities likely to be consumed with food are required to produce ill effects or cause the death of human beings or experimental animals. However, benzoic acid, one of the least harmful of food preservatives, should be avoided by persons with gastro-intestinal or renal diseases (7). Many attempts have been made to prepare compounds related to benzoic acid with even greater margins of safety in the preservation of food. For the purpose of comparing the effect of benzoic acid with that of certain of its substitution derivatives, a preliminary study of the acute toxicity of these compounds for white rats was undertaken.

From the School of Pharmacy, University of 'yland, Baltimore, Md.

'sented to the Scientific Section of the A. PH. A., l meeting, 1941.

Wm. R. Warner Fellow, 1939–41, University of 'yland.

EXPERIMENTAL

A portion of the results of this work is the subject of this report. For the comparison of the value of the LD_{50} for white rats found in this investigation with the statements on the toxicity of this substance for human beings and various experimental animals reported in the literature, Table I was drawn up. These other results do not include a measure of the standard deviation and, therefore, the significance of the differences of the reported values cannot be calculated.

Table I.—Summary of the Investigations of the Acute Toxicity of Benzoic Acid

Subject	Dose	Effect
Dogs, cats, rabbits[a]	2 Gm./Kg.	Lethal dose
Rabbits (dry-fed)[a]	1.7 Gm./Kg.	Lethal dose
Rabbits (fasting)[a]	1.52 to 1.83 Gm./Kg.	Lethal dose
Guinea pigs (intraperitoneal)[a]	1.4 Gm./Kg.	Lethal dose
White rats (intravenous)	1.714 ± 0.037 Gm./Kg.	LD_{50}
Human[b]	1 to 1.5 Gm. (capsule)	Gastric pain, nausea and vomiting

[a] See reference (8).
[b] See reference (9).

The method of determining the acute LD_{50} reported here for white rats consisted in injection of an aqueous solution of sodium benzoate of appropriate concentration into the saphenous vein at a rate of 1 cc. per min. Five doses were employed in each test, the first of which was likely to kill none of the animals injected and the last of which was likely to kill all of the animals injected. The doses differed by a constant ratio, the logarithm of which was 0.07; and varied from 1.2 to 2.29 Gm./Kg. Each dose was administered to a group of five rats weighing from 90 to 150 Gm. The solutions employed were of such concentration (12% to 23%) that the dose, based on the weight of the rat, would be contained in about 1 cc. The solutions were prepared by dissolving the highly purified acid in a solution of an equivalent weight of sodium carbonate. Results were recorded hourly for 5 to 6 hrs. to ascertain rate of onset of action, symptoms, etc., and the LD_{50} calculated from the number of animals dead in 24 hrs.

The symptoms of benzoic acid poisoning observed during the course of these experiments were those resulting from a heightening of the reflexes of the central nervous system, and consisted of tremors, clonic and often tetanic convulsions, with death occurring during a remission. These symptoms appeared within a few minutes after the injection and progressed rapidly with deaths often occurring one-half to one hour after injection. Surviving animals generally showed none of the nervous manifestations, but salivation, vomiting and diarrhea were present. A marked diuresis was seen in some cases.

For the calculation of the LD_{50} from these experiments, the use of the maximum likelihood method for the statistical treatment of biological results would be very cumbersome and would likely fail to give values much different from those obtained by a less exacting method. Therefore, the LD_{50}'s were calculated by Kärber's method (10); and the standard deviation of the individual determinations was obtained after first fitting the observed mortalities to a straight line by a method developed by Irwin and Cheeseman (11). The weighted mean of the values thus obtained in three separate determinations was calculated. The standard error of the mean was then obtained in the usual manner. In a different set of experiments carried out in the same way and on the same days, the toxicity of a fluorine substituted benzoic acid was studied. The results of these experiments are summarized in Table II.

TABLE II

Benzoic Acid	p-Fluorobenzoic Acid
LD_{50} ± S. D., Gm./Kg. (P = 0.66)	
1.686 ± 0.105	1.982 ± 0.137
1.855 ± 0.076	1.687 ± 0.109
1.630 ± 0.062	1.434 ± 0.055
Weighted Mean (LD_{50} ± S. E.), Gm./Kg.	
1.714 ± 0.062	1.542 ± 0.107
(P = 0.66)	(P = 0.66)
1.714 ± 0.124	1.542 ± 0.214
(P = 0.95)	(P = 0.95)

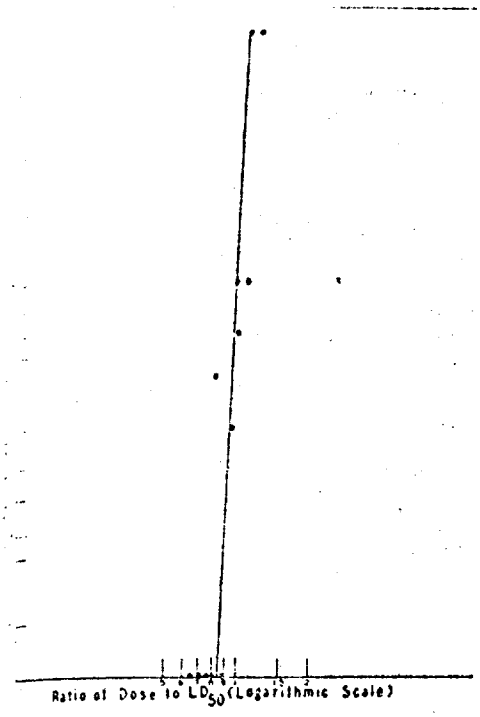
In experiments of this type in which a small number of animals and a large number of doses are employed, it has been shown that the values obtained by the maximum likelihood method of calculation and by the method outlined above are very close (11).

By plotting the percentage mortalities observed in an individual determination, as ordinates, against the corresponding doses expressed as a logarithm of the ratio of the doses to the LD_{50} calculated from the experiment, the results of the three experiments can be combined in a single curve, the regression line. This can be conveniently done by plotting the percentage mortalities against the ratio on logarithmic probability paper (Fig. 1). Seventy-five animals were employed in the construction of this curve, and its steepness indicates a great uniformity in the response of white rats to benzoic acid.

The significance of the differences between the individual determinations and between the mean values was calculated by the formula

$$\frac{m_1 - m_2}{\sqrt{(S.\,E._1)^2 + (S.\,E._2)^2}}$$

where m is an individual LD_{50} or a weighted mean LD_{50} and $S.\,E.$ is the standard deviation of the corresponding individual LD_{50} or the standard error of the corresponding weighted mean LD_{50}. When the value of this quotient is 2 or greater, the difference between m_1 and m_2 is generally considered significant. That is to say that when the value is 2 or greater, the



gure 1.—Regression Line for the Action of Benzoic Acid on White Rats.

observed difference could occur through chance or through an error in sampling only once in twenty times; and the chances are twenty to one that a greater toxicity is to be assigned to the substance whose LD_{50} is m_1 than to that whose LD_{50} is m_2. The application of this formula showed that no significant difference exists between the three values of the LD_{50} determined for benzoic acid. Likewise was the difference between the LD_{50} found for benzoic acid and that found for *p*-fluorobenzoic acid shown to be insignificant. On this basis, one is justified only in saying that benzoic acid and *p*-fluorobenzoic acid were found in these experiments to be of the same toxicity even though the absolute value of the weighted mean LD_{50} of benzoic acid is greater than that of *p*-fluorobenzoic acid. The use of statistical methods is therefore seen to be of great value in the study of the relation of chemical structure and physiological activity from a quantitative standpoint.

SUMMARY

The toxicity of benzoic acid when administered as the sodium salt intravenously to white rats corresponds closely to that reported for other animals. The value of the LD_{50} found in this investigation is 1.714 ± 0.124 Gm./Kg. ($P = 0.95$).

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The Journal of Biochemistry, Vol. 43, No. 4, 1956

DENATURATION AND INACTIVATION OF ENZYME PROTEINS

IV. DENATURATION AND INACTIVATION OF CATALASE

By BUNJI HAGIHARA, TAKESHI SHIBATA*, ICHIRO SEKUZU, FUMIO HATTORI, TAKEYOSHI NAKAYAMA, MITSUHIRO NOZAKI, HIROSHI MATSUBARA, AND KAZUO OKUNUKI

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(Received for publication, March 26, 1956)

Using the proteolytic method (*1*) for the determination of the "ratio of denaturation" of partially denatured enzyme proteins, it has been confirmed that the inactivation of some crystalline enzymes, such as α-amylases of *Aspergillus oryzae* and *Bacillus subtilis*, proceeds in parallel with the denaturation of the protein in the duration of urea-, heat- and acid-treatment (*2, 3*). In the previous paper (*3*), it has been clearly established that *Aspergillus* (Taka)-α-amylase, denatured to the reversible state by an acid-treatment, was perfectly susceptible to the bacterial proteinase and the "ratio of denaturation" of the protein even in the reversibly denatured state could be measured as in the case of the irreversibly denatured proteins.

The amylases used in the previous reports (*1, 2, 3*) were very stable enzymes and thought to be composed of simple protein. In the present investigation, crystalline beef catalase which is comparatively less-stable and one of the conjugated proteins was used in order to confirm whether the "ratio of denaturation" of this protein could be determined accurately by the proteolytic method or not when the protein was treated with urea as a typical denaturing agent or benzoate as a perturbator (*8, 9*). Furthermore, the relation between denaturation and inactivation of the catalase was also investigated, when the enzyme was inhibited by the specific inhibitors such as cyanide, fluoride, azide, or hydroxylamine. Though many results have been reported as to the inhibition of catalase activity in the presence of the above reagents, it has never been ascertained whether these inactivations resulted from the

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denaturation of the enzyme protein with specific inhibitors or from a simple combination of the inhibitors at the essential radicals of the enzyme without alteration of the whole intramolecular structure—denaturation. Though some results of the above investigation have been reported briefly elsewhere (*4, 5*), more detailed experiments will be described showing that the specific inhibitors of heme protein affect only the activity of enzyme, but do not denature the enzyme protein.

MATERIALS

Crystalline Catalase—The enzyme was prepared from beef liver according to the slightly modified method of Kitagawa and Shirakawa (*6*) and recrystallized four times.

The stock solution of the catalase was prepared dissolving the crystals with 0.02 *M* Na_2HPO_4 and the pH of the solution was adjusted to 6.8 with 0.02 *M* KH_2PO_4. Approximately 0.4 or 0.2 per cent solution, prepared as above, was used rapidly for the treatment with urea or the specific inhibitors, respectively.

Denaturing Agents and Specific Inhibitors—Urea was used as a typical denaturing agent. To make clear the mechanism of action of so-called "perturbators" (*8*) against heme proteins, sodium benzoate and sodium salicylate were used. As specific inhibitors, potassium cyanide, sodium fluoride, sodium azide, and hydroxylamine were used. These reagents were all commercial preparations.

All of the above agents were dissolved in 0.02 *M* phosphate buffer solution and pH of the solutions were adjusted to 6.8.

Crystalline Bacterial Proteinase—Though BPR (proteinase of *Bac. subtilis R* (*5*)) was mainly used in the previous reports (*2, 3*), BPN' (proteinase of *Bac. subtilis N*) was used in the present investigation since it has become easily obtainable as a commercial preparation*. As a proteinase for the determination of the "ratio of denaturation" of proteins, BPN' is equal as usefully as BPR (*1*).

METHODS

Assay for Catalase Activity—When a test solution of the catalase was diluted with distilled water, the catalase was considerably inactivated even at cold. But the catalase was not inactivated, if the test solution was diluted with ice cold 0.02 *M* phosphate buffer solution of pH 6.8 containing 0.05 *M* sodium chloride. The activity of the catalase was measured according to the method of Euler and Josephson (*7*) as follows:

* Crystalline proteinase of *Bac. subtilis* has been prepared in a large scale under the authors' guidance at Nagase Ltd. (Amagasaki, Japan), and is now easily obtainable as a commercial preparation "Nagarse."

1.0 ml. of the diluted enzyme solution was pipetted into 50 ml. of 0.005 *N* hydrogen peroxide solution containing 0.1 *M* phosphate buffer of pH 6.8 maintained at 0°, and each 5 ml. of samples were pipetted from the reaction mixture into 2 ml. of 2 *N* sulfuric acid at the 1st, 4th, 7th, and 10th minute from the begining of the reaction. The remaining hydrogen peroxide in the above test solutions was titrated with 0.005 *N* potassium permanganate and the unimolecular reaction constants (k) were calculated, then the value at zero time was obtained by extrapolation. Since the relation between enzyme concentration and above k value at zero time showed a slight deviation from the linear relation if a wide rage of the enzyme concentration was tested, the value was corrected by a standard curve in order to show the one in proportion to concentration of active enzyme.

Determination of the "Ratio of Denaturation"—The main procedures were carried out similary to those described in the previous reports (*1, 2, 3*), but they were slightly modified in detail as follows: 1 ml. of the catalase solution (test solution) was added to 2 ml. of about 0.05 per cent solution of the bacterial proteinase containing 0.1 *M* phosphate buffer of pH 7.5 and incubated at 30° for 10 minutes in the case of urea-treatment or at 20° for 15 minutes in other cases. After the above incubation, 1 ml. of 1.6 *M* trichloroacetic acid (TCA) was added and the concentration of the digestion product soluble in 0.4 *M* TCA was measured by Folin's colour reaction as before (*1, 2*). In order to determine the value of a completely denatured sample, the same aliquot of the test solution was denatured by heating at 70° for 10 minutes and treated with the proteinase solution as above. The "ratio of denaturation" (RD) of catalase protein in a test solution was calculated as follows (*1*):

$$RD\ (\%) = \frac{OD_{test} - OD_{native}}{OD_{denat.} - OD_{native}} \times 100$$

Treatments with Various Agents—These procedures will be described in the each section of the results.

RESULTS

Experiment I

Inactivation and Denaturation in Urea-treatment

When about 0.2 per cent catalase solution of pH 6.8 containing 3.5 *M* urea was incubated at 30° the "ratio of inactivation" (RI) and the "ratio of denaturation" (RD) almost coincided with one another at every stage in the duration, but the latter values were slightly higher as shown in Table I and Fig. 1. If the activities were measured after the proteolysis, the "RI" coincided well with the "RD" measured at the same time (see Curve B and C in Fig. 1). It was supposed that the small difference between the "RI" measured without proteolysis (Curve A) and the "RD" (Curve C) might be due to either reactivation

TABLE I

Inactivation and the Proteolytic Susceptibility of Crystalline Beef Catalase in the Duration of Urea-treatment

Time of treat.	Proteolysis	Activity test: Activity ($k_1 \times 1/4000$), After dilution, Immediate	Activity test: Activity ($k_1 \times 1/4000$), After dilution, 24 hrs.	Activity test: "Ratio of inact." (%), After dilution, Immediate	Activity test: "Ratio of inact." (%), After dilution, 24 hrs.	Proteolysis: Folin-Colour (OD_{test})	Proteolysis: "RD" (%)
min.							
0	– +	0.100 0.099	0.100 0.097	0.0 1.0	0 3	($OD_{nat.}$) 0.025	0.0
20	–	0.093	0.096	8.0	3.5	0.056	9.5
50	– +	0.084 0.080	0.083 0.072	16.3 20	17.2 27.9	0.085	18.7
80	–	0.080	0.081	20	19.3	0.106	24.7
120	– +	0.075 0.070	0.074 0.061	25 30	26.0 38.9	0.124	30.3
210	– +	0.066 0.061	0.065 0.052	34 39	34.9 47.7	0.157	40.4
390	– +	0.052 0.045	0.051 0.035	48 55	48.6 65.4	0.211	56.8
Complete denaturation	– +	0		100		($OD_{den.}$) 0.352	100

About 0.4 per cent catalase solution containing phosphate buffer of pH 6.8 was mixed with the same volume of 7.0 *M* urea solution and the mixture was incubated at 30°. At each time interval in the duration of the incubation, 3 test solutions, 0.5 ml., 1 ml. and 1 ml., were pipetted from the above solution and the 1st 0.5 ml. was immediately diluted to 1/4100 with the cold salts solution by two steps in order to assay the catalase activity. The 2nd and 3rd test solutions were immediately mixed with 2 ml. of the proteinase solution, and incubated at 30° for 15 minutes. One of above digested solutions was diluted to the same degree as the 1st one in order to assay "the activity after the proteolysis" and the other solution was used for determination of the "ratio of denaturation."

of inactivated catalase by the dilution as in the case of acid-treatment of Taka-α-amylase (*3*) or slight digestion of the native catalase with pro-

teinase. Therefore, the activity of the diluted solutions was measured after 24 hours to see whether further reactivation or further loss in avtivity would occur. When the samples were diluted without proteolysis and kept at 10° for 24 hours, the avtivities (Curve A′) were not appreciably different from the corresponding activities measured immediately after dilution (Curve A). Therefore, it was difficult to conclude that the catalase inactivated once with above urea-treatment had been reactivated by dilution as in the case of Taka-α-amylase inactivated with acid-treatment. On the contrary, if the samples were diluted after mixing with the proteinase and kept at 10° for 24 hours, the activities (Curve B′) were much lower than the corresponding activities measured immediately after dilution (Curve B). This result showed that even an active molecule of catalase is slightly susceptible to the proteinase, though it is much more resistant than a denatured molecule of catalase.

Experiment 2

Action of Perturbators against Catalase

It has been reported that the absorption spectrum of hemoglobin was reversibly shifted by the action of some reagents such as benzoates or salicylates which were called "perturbators" by Holden *et al.* (*8*). T. Tomimura *et al.* (*9*) have also reported that these reagents somewhat affect the spectrum, catalatic and peroxidatic activities of horse liver catalase.

In the present investigation the "RI" and the "RD" of beef catalase were measured at the same time in the duration of teratment with 1.3 *M* benzoate at 20°. As shown by the results given in Fig. 2, the inactivation and denaturation proceeded similarly as in the case of urea-treatment. Therefore, benzoate is concluded to act against catalase as a denaturing agent under this experimental concentration.

The action of sodium salicylate was also investigated, but the "RD" could not be measured by the ordinary procedure since salicylate gave a strong colour reaction with Folin reagent and also showed strong extinction at 278 mμ. Therefore, the "RD" was calculated from the amount of precipitatable protein with 0.4 *M* TCA after the proteolysis —corresponding to remaining native protein— which was rather more difficult to measure accurately than in the case of a digestion-product in the ordinary procedure. However, it was confirmed that salicylate behaved in an almost identical way to benzoate.

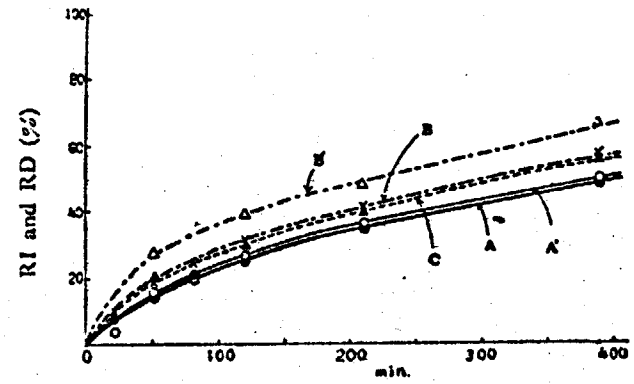

FIG. 1. Courses of inactivation and denaturation of catalase in the duration of urea-treatment.

Experimental condition; see Table I.

A Ratio of inactivation measured immediately after dilution.
A′ " " measured 24 hrs. after dilution.
B " " immediately after dilution following the proteolysis.
B′ " " 24 hrs. after dilution following the proteolysis.
C Ratio of denaturation measured by the proteolysis method.

Experiment 3

Action of Cyanide

As it is a well known fact that the activity of catalase is strongly inhibited by cyanide at the final concentration of 10^{-6} *M* in the solution of enzyme reaction, the relation between inactivation and denaturation of catalase was studied in the treatment with 10^{-2} *M* potassium cyanide. In the experiment, concentration of catalase was about 0.1 per cent which was the minimum concentration in order to determine the "RD" by the proteolytic method and the catalytic activity of the treated solution was measured after dilution about 2000 times for it is too strong to estimate the accurate activity. As the above diluted solution was further diluted 51 times with the substrate solution, the concentration of the inhibitor in the treated solutions was diluted about 10^5 times in the solution of enzyme reaction. On the other hand, the treated solution was diluted 3 times in the solution for determination of the "RD," since 1 ml. of the solution was mixed with 2 ml. of the proteinase solution. Therefore, concentration of the cyanide was 0.33×10^{-2} and $0.33 \times$

10^{-6} *M* in the solutions for determination of denaturation and about 10^{-7} and 10^{-11} *M* in the solutions for assay of catalase avtivity, though it was 10^{-2} *M* and 10^{-6} *M* respectively in the treated solution. The results of the experiment are shown in Table II. As shown in the Table, both denaturation and inactivation were not detected at any stage in the duration of the treatment with the cyanide even in the concentration of 10^{-2} *M*. Since the concentration of cyanide was sufficient to inhibit the enzyme in the treated solution, it was concluded that the enzyme was reactivated completely by dilution of the inhibitor in the process for assay of activity. In the process for determination of denaturation, the concentration of the inhibitor was also sufficient to inhibit the activity at least in the case using 10^{-2} *M* cyanide in the treated solution. Therefore, it was concluded from the results shown in the Table that cyanide inhibits the activity of catalase without denaturing the enzyme protein. Furthermore, it was shown from the results that the action of cyanide on catalase was not affected during the treatment at 30°.

Experiment 4

Action of Fluoride

The experiment was carried out as in the case of *Experiment 3*, except that sodium fluoride at a final concentration of 1×10^{-1} *M* was used. This concentration was quite sufficient to inactivate catalase completely. As shown in Table III, the same results were obtained as in the case of cyanide treatment.

Experiment 5

Action of Azide

The experiment was carried out as in the case of *Experiment 3*, except that sodium azide at a final concentration of 0.5×10^{-3} or 0.5×10^{-2} *M* was used. The former concentration of the azide was quite sufficient to inactivate catalase and even the latter concentration was believed to be sufficient to inactivate more or less completely. The results are shown in Fig 3. As shown in Fig. 3 the catalse was not denatured at all with this reagent even at the concentration of 0.5×10^{-3} *M*, similarly as in the case of cyanide or fluoride-treatment. However, considerably strong inactivation was observed even after dilution. Therefore, it seems that the catalase was inactivated by azide-treatment without denaturing the enzyme protein and the inactivated enzyme cannot be reactivated

by dilution of the reagent.

TABLE II

Inactivation and Denaturation of Catalase in the Duration of Treatment with Potassium Cyanide

Conc. of CN (*M*) / Time of treat. (*min.*)	0			10^{-6}			10^{-2}		
	Activity** (k)	OD_{test}*	OD_{test}*** $-OD_{nat.}$	Activity (k)	OD_{test}*	OD_{test}*** $-OD_{nat.}$	Activity (k)	OD_{test}*	OD_{test}*** $-OD_{nat.}$
0	0.029	0.073	0	0.029	0.073	0	0.030	0.074	0.001
15				0.028	0.065	−0.008	0.029	0.080	0.007
30				0.028	0.060	0.003	0.026	0.070	−0.003
50				0.028	0.076	0.003	0.029	0.065	−0.008
80				0.028	0.075	0.002	0.029	0.066	−0.007
120	0.029	0.070	−0.003	0.029	0.076	0.003	0.030	0.070	−0.003
Complete denaturation	0	0.234	0.161	0	0.236	0.163	0	0.241	0.168

About 0.2 per cent solution of crystalline beef catalase was mixed with the same volume of buffer solution of pH 6.8 containing 0, 2×10^{-6} and 2×10^{-2} *M* KCN, and the mixtures were kept at 30°. Two test solutions, 0.5 ml. and 1 ml. were pipetted from above solutions at the same time at each time interval in the duration of the incubation. The former soln. was used for assaying the activity after dilution 1700 times and the latter was used for the determination of denaturation by the proteolytic method.

* Complete denaturation was carried out by heating an liquot of the treated solution at 70° for 10 minutes.

** The activity was represented by the unimolecular velocity constant (k) obtained by the diluted enzyme solution.

*** OD_{test} and $OD_{nat.}$ are the optical densities of Folin colour-solutions of the filtrates obtained from the digested solution of the treated samples and non-treated samples, respectively, after addition of TCA at a final concentration of 0.4 *M*. Therefore, the values OD_{test}-$OD_{nat.}$ show the amount of denatured protein measured by the proteolytic method.

TABLE III

Inactivation and Denaturation of Catalase in the Duration of Treatment with Sodium Fluoride

Conc. of F^- (M) / Time of treatment (min.)	0			10^{-1}		
	Activity (k)	OD_{test}	$OD_{test}-OD_{nat.}$**	Activity* (k)	OD_{test}	$OD_{test}-OD_{nat.}$**
0	0.030	0.065	0.000	0.029	0.060	−0.005
30	0.030	0.068	0.003	0.030	0.050	−0.015
100	0.029	0.073	0.008	0.031	0.052	−0.013
Complete denaturation	0.000	0.200	0.135	0.000	0.190	0.125

The experiment was carried out similarly to *Expt. 3* (Table II) except that 10^{-1} *M* sodium fluoride was used instead of potassium cyanide.

* The activity was represented by the unimolecular velocity constant obtained by 1200 times diluted solutions of treated solution.

** The values show the amount of denatured protein.

Experiment 6

Action of Hydroxlamine

The experiment was carried out as in the case of *Experiment 3*, except that hydroxylamine at a final concentration of 2×10^{-3}, 1×10^{-3}, or 4×10^{-4} *M* was used in the treated solution. As shown in Fig. 4, the same results were obtained as in the case of azide.

Experiment 7

Coupled Action of Denaturing Agent and Specific Inhibitor

Since the catalases treated with the above four specific inhibitors were still as resistant to the proteinase as untreated one, it was supposed that the secondary intramolecular structure of catalase was not affected by them. In order to confirm this supposition more clearly, the catalase was treated with one of above inhibitors, sodium azide, in the presence of urea. The time-courses of inactivation and denaturation were shown in Fig. 5 when the catalase was treated with 4 *M* urea alone, 0.5×10^{-4} *M* sodium azide alone and both. As shown in Fig. 5, the course of denaturation of the catalase in the urea treatment (Curve *A*) was not

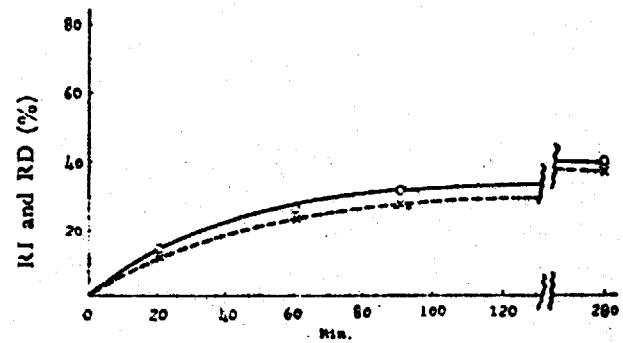

FIG. 2. The action of sodium benzoate on catalase.

About 0.2 per cent catalase solution containing phosphate buffer of pH 6.5 was mixed with the same volume of 1.86 *M* sodium benzoate and the mixture were kept at 20°. The "ratio of inactivation" and "ratio of denaturation" were measured, as in the case of urea-treatment, at each time in the duration of above incubation (see Table I), except that activity of the treated solution was measured after a dilution of approximately 2000 times.

—○— the ratio of inactivation

---×--- the ratio of denaturation

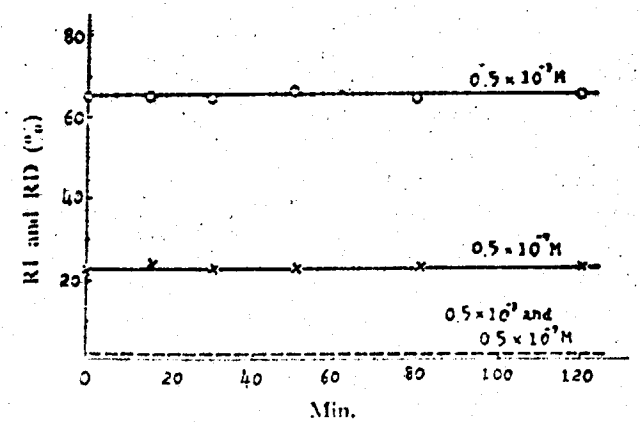

FIG. 3. The inhibition of catalase by sodium azide.

The experimental procedure was similar to *Expt. 3* except that 0.5×10^{-4} *M* and 0.5×10^{-7} *M* sodium azide were used in the treated [illegible] instead of cyanide (see Table II).

Solid lines: the ratio of inactivation.

Broken lines: the ratio of denaturation.

The assay of catalase activity was carried out after 1,600 times dilution. Temp. 20°.

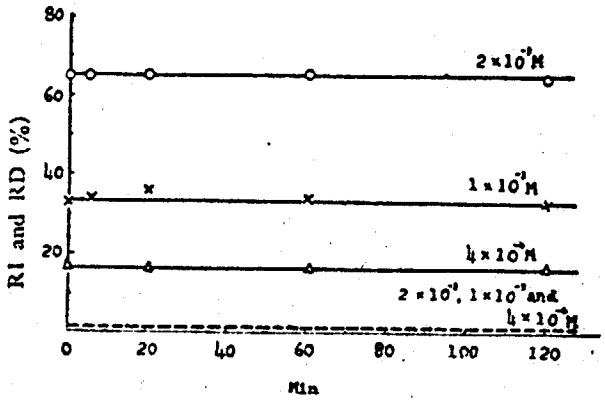

FIG. 4. The inhibition of catalase by hydroxylamine.

The experiment was carried out as *Expt. 3*, except that 2×10^{-3} and 4×10^{-4} *M* hydroxylamine were used instead of cyanide (see Table II).

Solid line: the ratio of inactivation.

Broken line: the ratio of denaturation.

The assay of catalase activity was carried out in a similar manner to that in Fig. 3.

affected by the addition of the azide (Curve C), notwithstanding that the course of inactivation was strongly changed (see Curves a and c). Furthermore, the difference between the "RI" of catalase treated with urea alone (Curve a) and that treated with both urea and azide (Curve c) was almost the same as the value of azide inactivation in the absence of urea (Curve b). From the above results, it was concluded almost certainly that the catalase was not affected by the azide as regards its intramolecular secondary structure.

DISCUSSION

From the construction of the heme protein, many agents which modify the activity of heme enzymes may be classified into following three groups: 1) Specific inhibitors which inactivate the heme enzyme by combining with the prosthetic group (heme) alone without any influence to the secondary intramolecular structure of the protein moiety; 2) Specific inhibitors which combine with heme alone but cause the alteration of the protein moiety by the influence of the above

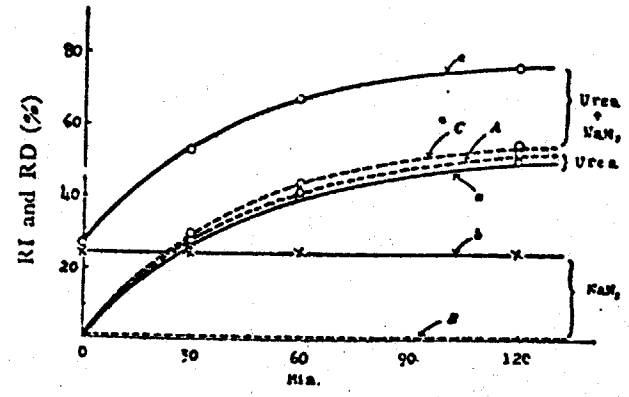

FIG. 5. Effect of sodium azide on the inactivation and denaturation of catalase being treated with urea.

Three different solutions containing 4 *M* urea alone (Curves A and a), 0.5×10^{-4} *M* sodium azide alone (Curves B and b) and both 4 *M* urea and 0.5×10^{-4} *M* sodium azide (Curves C and c) were incubated at 20°. The "RI" and "RD" of the 3 solutions were measured at several stages in the duration of the incubation similarly to the case of urea-treatment (see Table I). In the figures, solid lines (indicated by capital letters) show the courses of inactivation and broken lines (indicated by small letters) show the courses of denaturation.

combination, and thus cause denaturation of the protein moiety; 3) Ordinary denaturing agents which act on the protein moiety alone and cause inactivation of the enzyme by the alteration of the secondary intramolecular structure—denaturation—of the protein.

The first is further divided into two groups, one of which combine quite reversibly with heme and the other which does not combine reversibly. From the results of *Experiments 3–6*, it was concluded that cyanide and fluoride belong to the former group and azide and hydroxylamine belong to the latter. Though it may not be neglected from only these results that the above inhibitions might cause some slight modification of the structure of the protein moiety which could not detected by the proteolytic method, this posibility was almost completely denied by the result of *Experiment 7* in which the time course of denaturation in the urea-treatment was not influenced by the addition of one of the above inhibitors, sodium azide.

The third group may also be divided into two groups, irreversible

and reversible denaturing agents, as in the case of the first group. Urea acted as an almost completely irreversible denaturing agent in the condition used in *Experiment 1*. Though the "perturbators" such as benzoate and salicylate have been reported as reversibly modifing agents of the spectrum of heme proteins (*8, 9*), above agents acted on catalase quite similarly to the urea in the *Experiment 2*. As the recovery of the spectrum of heme protein may not always represent the renaturation of the protein molecule, it was concluded that the agents have behaved as ordinary protein-denaturing agents in the conditions employed. However, the possibility cannot be excluded that the perturbators will act as reversibly denaturing agents when other conditions, especially an other concentration, is employed.

Recently Goldacre and Galston (*10*) have reported the assumption that the specific inhibition of catalase with the substituted phenols was due to direct combination with catalase (heme)-hydogenperoxide complex rather than to nonspecific denaturation of the protein. As the perturbators used in the present investigation have a structure analogy with the above phenol derivatives, it is probable that the same mechanism as above may take place in the denaturation of catalase with the perturbators, i.e., the denaturation may be caused by the influence of the combination of the agents with heme moiety. If the above mechanism is true, the perturbators must belong to the second group mentioned above. However, the above problem could not be solved by the present investigation.

SUMMARY

By assaying the activity and determining the denaturation by the proteolytic method at the same time, the relation between inactivation and denaturation of crystalline beef catalase was investigated in the duration of treatment with urea, perturbators, and four specific inhibitors. The "ratio of inactivation" (RI) and the "ratio of denaturation" (RD) of the catalase almost coincided with one another at any stage in the duration of urea-treatment, but the "RD" was always slightly larger than the "RI" because catalase even native, was slightly digested by the proteinase. So called "perturbators," such as benzoate and salicylate, behaved similarly to urea. Cyanide and fluoride inhibited the enzyme activity without denaturation of the enzyme protein and the inactivated enzyme was completely reactivated by the dilution. Azide and hydroxylamine also inactivated the enzyme without denaturation, but the inactivated enzyme was not reactivated by the dilution. As the time course of denaturation of catalase in the presence of urea was not changed by the addition of azide, it was concluded that the above specific inhibitors have not affect at all on the secondary intramolecular structure of protein moiety of catalase molecule.

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17278. Effect of Administration of Sodium Benzoate on the Level of Glutamine in Blood. VI.

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In previous publications we reported on the effect of the administration of insulin, glucose,[1] amino acids and sodium bicarbonate[2] on the level of glutamine in the blood. It was suggested that the effect of insulin in lowering the level of glutamine in the blood might be due, in part, to the depression of oxidative deamination of amino acids by insulin. Since it has been reported that benzoic acid depresses the oxidation of d-amino acids[3] and other intermediary metabolites[4,5] the effect of its administration on the level of glutamine was investigated.

Procedure and methods. Male rabbits in the post absorptive state were used for these studies. The effect of the administration of sodium benzoate was studied also in a normal human female subject. The benzoate was administered either intravenously or orally as indicated in the table. Blood was collected in tubes containing sodium oxalate and the plasma separated by centrifugation. Glutamine was determined by the method described in a previous publication.[6] The total amino acids were determined by the method of Simonelli.[7] The blood sugar (total reducing substance) was determined by the Folin-Wu method[8] which includes other non-glucose reducing substances. In some cases the blood was allowed to glycolize at 37°C overnight in order to determine the change in non-glucose reducing substances.

Observations. Doses of 0.25 g, 0.5 g, and 0.75 g of benzoic acid neutralized with sodium hydroxide and injected intravenously produced a fall in the level of glutamine in the plasma both in the 30 min. and 60 min. period after injection. The extent of the fall varied in different animals and was not related to the size of the dose of benzoic acid. Statistical analysis of the fall showed that the drop was statistically significant for both periods (see table).

The level of the total amino acid nitrogen in the blood plasma tended to fall in all animals; however, this was negligible in some animals. The extent of the change in the level of glutamine was not parallel with the changes in total amino acid nitrogen. The blood sugar (total reducing substances) also rose in all the animals which received benzoic acid intravenously. The extent of the rise also was variable and two of the animals which showed the most marked rise (Rabbits nos. 1 and 3) in the "blood sugar" received the smallest dose of benzoic acid. By incubating the blood at 37°C overnight it was found

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rabbit and hippuric acid in man. If this is so, it would mean that the level of glutamine is depressed irrespective of the mode of detoxification used by the animal organism. It is interesting to note the variable effect of the administration of benzoic acid on the level of the total amino acids and the total reducing substances in the blood. Although glycine was withdrawn from the metabolic mixture as hippuric acid in the experiment on the human subject (several grams of crystalline hippuric acid were isolated from her urine), still the level of the total amino acids in the blood was lowered to no greater extent than in some of the rabbits. These variable effects in the different animals suggest that variable metabolic changes, besides that involved in the formation of benzoyl glycuronide, probably occur in different rabbits. The fall in the level of glutamine may be due, in part, to the inhibition of the oxidative deamination of amino acids beside the inhibition of other oxidative processes.[4,5] This would reduce the supply of nitrogen for amidation. However, where there was a fall in the level of the total amino acids in the blood it is possible that certain amino acids were withdrawn from the metabolic mixture thus reducing the supply of nitrogen for amidation. The inhibition of deamination alone should tend to raise the level of the amino acids in the blood. Since in some cases the level of glutamine was depressed without any significant change in the level of the total amino acids (Rabbits nos. 2, 3 and 7) it is possible that the process of amidation was directly depressed.

Christensen and his co-workers[9] claim that special mechanisms are present in cells for concentrating amino acids and that in the presence of high concentrations of amino acids there is competition between the various amino acids for these mechanisms. If the depression in the level of glutamine in the plasma were due to a shift of glutamine into tissue cells due to the removal of glycine by conjugation with benzoic acid then one should obtain a significant drop in the level of the total amino acid nitrogen in the plasma. This did not occur in a number of experiments. Furthermore, the statistical analysis indicated that the changes in the level of amino acid nitrogen were not significant while those for glutamine were highly significant (see additional discussion in previous paper and table). This problem requires further study.

It is of interest to note that the smaller doses of sodium benzoate (Rabbits nos. 1 and 3) produced the most pronounced rise in blood sugar (total reducing substances). The reason for this effect is not apparent at the present time.

Summary and conclusions. 1) Sodium benzoate administered intravenously and orally to rabbits and one human subject was found to depress the level of glutamine in the blood plasma.

2) The intravenous administration of sodium benzoate produced a rise in the total reducing substances in the blood which was highest with the smallest doses of benzoate.

3) The changes in the glutamine level were not related to either the change of the total amino acid level or the level of total reducing substances in the blood.

4) It is suggested that amidation may be either directly or indirectly depressed by the administration of sodium benzoate.

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Received July 18, 1949. P.S.E.B.M., 1949, 71.

TABLE I.
Effect of Administration of Sodium Benzoate on the Level of Glutamine in the Blood.

Rabbit No.	Wt., kg	Substance administered*	Time, min.	Glutamine in plasma, mg per 100 ml.	Amino acid nitrogen in plasma, mg per 100 ml.	Blood sugar, mg per 100 ml
1	2.5	0.25 g benzoic acid in 8 ml water	0	8.0	9.4	132
			30	6.3	8.7	155
			60	6.8	9.1	220
2	2.6	0.25 g benzoic acid in 8 ml water	0	7.9	10.0	115
			30	5.9	9.7	124
			60	6.0	10.1	152
3	2.9	0.25 g benzoic acid in 5 ml water	0	8.6	9.9	126
			30	6.5	9.8	218
			60	6.6	9.2	260
4	2.9	0.5 g benzoic acid in 10 ml water	0	7.5	10.5	118
			30	6.5	9.8	145
			60	7.5	10.0	172
5	2.9	0.75 g benzoic acid in 10 ml water	0	8.0	8.0	113
			30	7.3	7.3	122
			60	6.9	6.9	119
6	2.9	0.75 g benzoic acid in 10 ml water	0	8.3	7.8	122
			30	7.3	7.3	134
			60	6.9	7.3	153
7	4.2	2.0 g in gelatin capsules + 15 ml water (orally)	0	8.1	7.2	—
			75	5.7	7.1	95
			175	6.1	7.4	96
Human subject 59.1		5.9 g sod. benzoate in capsules + 200 ml water	0	6.6	9.1	89
			45	7.0	8.5	77
			85	6.0	8.4	84
			135	4.8	8.7	92
8		No treatment	0	8.3	9.0	
			30	8.4	9.1	
			60	8.1	9.4	
		Saline controls, avg. for 6 animals see previous paper	0	6.4	6.3	101
			30	6.5	6.2	105
			60	6.5	6.2	109

* Sodium benzoate was administered intravenously except where otherwise indicated.

Statistical analysis of the effect of the intravenous administration of sodium benzoate gave a Fisher "t" value of 6.64 and 6.30, with $P < 0.01$ for the changes in the level of glutamine for the 30 and 60 min. periods respectively, which is highly significant. The corresponding changes in the level of amino acid nitrogen gave a Fisher "t" value of 2.03 with $P > 0.05$ and 1.81 with $P > 0.1$ which were not statistically significant.

in some of the experiments that the rise was due in part to an increase in a reducing substance which did not undergo glycolysis and was undoubtedly due to glycuronic acid.

Sodium benzoate was administered orally in capsules to one rabbit and one human female subject in doses of 2.0 g and 5.9 g respectively. The level of glutamine dropped markedly in both cases. However, the level of amino acid nitrogen remained practically unchanged in the rabbit. In the human subject the glutamine level dropped 27% and the total amino acid nitrogen only about 4% at the end of 135 min. after the administration of sodium benzoate. The "blood glucose" showed very little change in both cases and in fact was slightly lower in the human subject after 45 minutes.

Discussion. It is claimed that benzoic acid is detoxified by forming a glycuronide in the

rabbit and hippuric acid in man. If this is so, it would mean that the level of glutamine is depressed irrespective of the mode of detoxification used by the animal organism. It is interesting to note the variable effect of the administration of benzoic acid on the level of the total amino acids and the total reducing substances in the blood. Although glycine was withdrawn from the metabolic mixture as hippuric acid in the experiment on the human subject (several grams of crystalline hippuric acid were isolated from her urine), still the level of the total amino acids in the blood was lowered to no greater extent than in some of the rabbits. These variable effects in the different animals suggest that variable metabolic changes, besides that involved in the formation of benzoyl glycuronide, probably occur in different rabbits. The fall in the level of glutamine may be due, in part, to the inhibition of the oxidative deamination of amino acids beside the inhibition of other oxidative processes.[4,5] This would reduce the supply of nitrogen for amidation. However, where there was a fall in the level of the total amino acids in the blood it is possible that certain amino acids were withdrawn from the metabolic mixture thus reducing the supply of nitrogen for amidation. The inhibition of deamination alone should tend to raise the level of the amino acids in the blood. Since in some cases the level of glutamine was depressed without any significant change in the level of the total amino acids (Rabbits nos. 2, 3 and 7) it is possible that the process of amidation was directly depressed.

Christensen and his co-workers[9] claim that special mechanisms are present in cells for concentrating amino acids and that in the presence of high concentrations of amino acids there is competition between the various amino acids for these mechanisms. If the depression in the level of glutamine in the plasma were due to a shift of glutamine into tissue cells due to the removal of glycine by conjugation with benzoic acid then one should obtain a significant drop in the level of the total amino acid nitrogen in the plasma. This did not occur in a number of experiments. Furthermore, the statistical analysis indicated that the changes in the level of amino acid nitrogen were not significant while those for glutamine were highly significant (see additional discussion in previous paper and table). This problem requires further study.

It is of interest to note that the smaller doses of sodium benzoate (Rabbits nos. 1 and 3) produced the most pronounced rise in blood sugar (total reducing substances). The reason for this effect is not apparent at the present time.

Summary and conclusions. 1) Sodium benzoate administered intravenously and orally to rabbits and one human subject was found to depress the level of glutamine in the blood plasma.

2) The intravenous administration of sodium benzoate produced a rise in the total reducing substances in the blood which was highest with the smallest doses of benzoate.

3) The changes in the glutamine level were not related to either the change of the total amino acid level or the level of total reducing substances in the blood.

4) It is suggested that amidation may be either directly or indirectly depressed by the administration of sodium benzoate.

[9] Christensen, H. N., Streicher, J. A., and Elbinger, R. L., *J. Biol. Chem.*, 1948, **172**, 515.

Received July 18, 1949. P.S.E.B.M., 1949, 71.

J. Dairy Sci. 25: 168-174, 1942.

REPORT OF A STUDY ON THE TOXICITY OF SEVERAL FOOD PRESERVING AGENTS[1]

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The deterioration of some foods caused by the activity of microorganisms may be prevented and the keeping quality of the foods greatly improved by the addition of certain chemical compounds. It is obvious that preservatives which produce a marked toxic effect should not be used in food intended for human consumption. On the other hand, even when a marked toxicity is not shown, the consumption of food containing unusual quantities of preservatives may be dangerous and harmful because of the hidden cumulative effect.

Definite proof of toxicity lies in knowing how the chemical agent is handled in the body. By tracing the chemical reactions of the preserving agent in the animal body and determining the method by which it is eliminated from the body, an accurate statement concerning its toxicity can be made.

This experiment was designed to yield information that may contribute to the knowledge of the toxic properties of certain chemical substances used in human food, and a bactericidal material used for sterilizing utensils. The amount of gain made by growing white rats was used as an indication of the toxic effects. Individual feeding trials were conducted with diets to which were added calcium propionate, sodium propionate, sodium benzoate, zephiran, and sodium benzoate plus glycine. Propionates are used in the bread industry and to some extent in packaged cheese in order to control mold. Zephiran is a new germicide which may be used for disinfecting dairy utensils. Sodium benzoate has been used for some years as a preservative for various food products.

Griffith (1) has shown that when 1.5 per cent or more sodium benzoate was in the diet, the rate of growth in young rats was decreased. The excretion of hippuric acid in the urine accounted for 66 to 95 per cent of the benzoic acid consumed as sodium benzoate. Small doses of sodium benzoate gave nearly perfect quantitative recovery of hippuric acid. As the amount of sodium benzoate was increased, the percentage recovered as hippuric acid decreased. It was assumed that this showed a lack of sufficient glycine in the animal for the detoxication of the benzoic acid. The addition of glycine

Received for publication October 14, 1941.

[1] This study was a graduate research problem conducted under the direction and supervision of Professor T. S. Hamilton, Department of Animal Husbandry, University of Illinois.

to the diet containing sodium benzoate increased the growth rate and decreased the toxic effects.

Rittenberg and Schoenheimer (4) showed that animals use tissue glycine in preference to dietary glycine for the detoxication of benzoic acid. About two-thirds of the nitrogen in the hippuric acid excreted was not of dietary origin. An excess of dietary glycine did not change this ratio.

PLAN OF EXPERIMENT

White rats were fed diets containing calcium propionate, sodium propionate, sodium benzoate, zephiran, and sodium benzoate plus glycine. Feeding of the experimental diets started when the rats were about four weeks of age and continued for four to five weeks. The paired-feeding technique (2, 3) was used, with a modification so that one control animal was compared with two experimental animals. Eight triplicates (24 rats) were fed during each part of the experiment. In each triplicate, litter mates of the same sex were used and the maximum weight variation of the young rats at the start was 4 grams.

In series I the control animals were fed a basal diet containing calcium lactate, a second group was fed diets containing 1 per cent and 3 per cent calcium propionate, and a third group was fed diets containing 1 per cent and 3 per cent sodium propionate.

In series II the control diet contained calcium lactate, one experimental diet contained 3 per cent sodium benzoate, and the other experimental diet contained 3 per cent zephiran, a germicidal agent. Zephiran is sold as a ten per cent aqueous solution of a mixture of high molecular alkyldimethylbenzylammonium chlorides. When used as a rinse for utensils, 1 part of zephiran is added to 5,000 to 10,000 parts of water.

In series III the control diet previously used was compared with an experimental diet containing 1 per cent sodium benzoate and another containing 3 per cent sodium benzoate plus glycine. Theoretically, .52 gram of glycine is required to detoxify 1 gram of sodium benzoate, but one gram of glycine was fed for each gram of sodium benzoate in order to supply more glycine than was needed.

In series IV all the rats used in series III were fed a diet containing 3 per cent sodium benzoate. The same grouping was maintained in order to study the residual effect of feeding small quantities of sodium benzoate and also of feeding glycine.

The composition of the diets used in each series is given in table 1. Waste by rats was prevented by mixing enough distilled water with the feed at feeding time to give the mixture the consistency of paste. Distilled water was supplied at all times in individual water fountains.

RESULTS

Comparisons of control rats with experimental rats showed that calcium

propionate, sodium propionate, and zephiran fed at the levels used in these trials did not decrease the amount of gain. Sodium benzoate fed at the rate of 3 per cent proved harmful: the amount of gain was much less than for the control group, and two rats out of eight died before the end of the trial.

TABLE 1

Composition of diets

Series and group	Constituents of each diet					
	Fox chow	Yeast	Cod-liver oil	Salt	Calcium lactate	Experimental substance
	%	%	%	%	%	%
Series Ia:						
Control	91.5	5.0	2.0	0.5	1.0	.....
Ca propionate	91.5	5.0	2.0	0.5	.....	1.0
Na propionate	91.5	5.0	2.0	0.5	.....	1.0
Series Ib:*						
Control	89.5	5.0	2.0	0.5	3.0	.....
Ca propionate	89.5	5.0	2.0	0.5	.....	3.0
Na propionate	89.5	5.0	2.0	0.5	.....	3.0
Series II:						
Control	89.5	5.0	2.0	0.5	3.0	.....
Na benzoate	89.5	5.0	2.0	0.5	.....	3.0
Zephiran	89.5	5.0	2.0	0.5	.....	3.0
Series III:						
Control	89.5	5.0	2.0	0.5	3.0	.....
Na benzoate	91.5	5.0	2.0	0.5	.....	1.0
Na benzoate plus glycine	89.5	5.0	2.0	0.5	.....	3.0‡
Series IV:†						
Na benzoate	89.5	5.0	2.0	0.5	.....	3.0

* Rats continued from series Ia.
† Rats continued from series III.
‡ One part of glycine was added for each part of sodium benzoate.

For the group of rats receiving 3 per cent sodium benzoate, the average amount of gain produced per 10 grams of feed consumed was 1.01 grams smaller than the average for the control group. This is considered a significant margin. The addition of glycine to the 3 per cent sodium benzoate diet reduced the toxic effect of the benzoic acid. The difference between the amount of gain produced per 10 grams of feed consumed when glycine was fed was only .24 gram in favor of the control group. This fact is probably significant, however, because the gain of the control individual over that of the rat receiving sodium benzoate plus glycine was consistent for all pairs tested. This result may indicate a limited capacity of the animal organism to synthesize glycine or to utilize dietary glycine for the detoxication of sodium benzoate.

TABLE 2

Grams of gain produced

Series and groups	Gain by triplicates								Total gain	Average	Difference†	S.D.	P. value	Odds	Gain/10 gms. feed
	1	2	3	4	5	6	7	8							
Series Ia (4 wks.)															
Control group	88	82	70	86	88	60	78	80	632	79.0	.....	.....	.....	.....	3.34
Ca propionate	90	82	66	87	91	62	82	80	640	80.0	+ 1.0	2.29	.1429	1: 7	3.38
Na propionate	85	84	67	86	87	67	80	82	638	79.8	+ .75	2.11	.1881	1: 5	3.37
Series Ib (3 wks.)															
Control group	50	26	30	50	43	20	33	36	288	36.0	.....	.....	.....	.....	1.74
Ca propionate	52	16	34	50	39	17	36	35	279	34.9	− 1.12	4.25	.2586	1: 4	1.68
Na propionate	53	17	35	51	36	25	43	33	293	36.6	+ .63	6.08	.3994	1: 3	1.77
Series II (4 wks.)															
Control group	63	70	65	74	69	65	74	77	577	69.6	.....	.....	.....	.....	3.24
Na benzoate (3%)	51	53	58	*	*	59	32	32	285	47.5	−21.5	15.98	.0148	1: 68	2.23
Zephiran (3%)	*	71	70	76	80	62	77	73	509	72.7	+ 2.1	4.67	.1525	1: 7	3.35
Series III (5 wks.)															
Control group	82	100	98	103	97	100	98	98	776	97.0	.....	.....	.....	.....	3.12
Na benzoate (1%)	87	96	98	97	95	97	97	102	769	96.1	− .88	3.55	.2666	1: 4	3.09
Na benzoate (3%) plus glycine	74	87	90	94	95	92	90	95	717	89.6	− 7.38	3.23	.0002	1: 5000	2.88
Series IV‡ (2 wks.)															
Group 1	18	22	*	23	19	26	28	26	162	23.1	.....	.....	.....	.....	1.33
Group 2	32	20	28	27	23	33	22	29	214	26.8	+ 3.43	5.90	.1033	1: 10	1.55
Group 3	32	24	29	*	25	32	30	22	194	27.7	+ 4.33	5.47	.0668	1: 15	1.61

* Omitted because of incomplete results.

† Mean difference between control group and experimental group.

‡ Rats of group 1 continued from control group of series III.
Rats of group 2 continued from Na benzoate (1%) group of series III.
Rats of group 3 continued from Na benzoate (3%) plus glycine group of series III.

At the termination of the tests in series III, all the rats of that trial were changed to a diet containing 3 per cent sodium benzoate. Results obtained from this diet were compared with those of previous diets fed, but the probable error was large. The control group which had never received any sodium benzoate was most seriously affected by the change in diet. This group consumed 7.49 grams of feed per gram of gain compared to 6.44 for the group which had previously received 1 per cent sodium benzoate and 6.20 for the group which had previously received 3 per cent sodium benzoate plus glycine.

These results indicate that the group of rats which had previously received glycine had more resistance to the toxic effect of sodium benzoate than any other group. The utilization of stored glycine may explain the increased resistance. However, it seems reasonable to assume that the glycine reserve of group 2, which had received previously sodium benzoate and no glycine, should have been depleted and that additional quantities of sodium benzoate should have been very toxic. Instead, this group of rats made a greater gain than group 1 and practically as much gain as group 3. This fact may indicate that with limited feeding of sodium benzoate a mechanism was set up which enabled the animals to more readily combat toxicity.

Series II gives further evidence that rats which consume sodium benzoate develop a resistance to its harmful effects. The symptoms of toxicity were evident and the deaths caused by it occurred 7 to 20 days after the start of the trial. The rats which lived longer than 20 days showed an increasing resistance to the toxic effects of sodium benzoate.

The toxicity of sodium benzoate was expressed by definite, characteristic symptoms. The animal first became irritable and rigid, responding to the slightest touch or disturbance by biting the cage or the handler. As the symptoms progressed, it lost its power of coordinated movements and had convulsions.

A summary of the grams of gain produced in each part of this experiment is presented in table 2.

SUMMARY

A study was made of the amount of gain produced in growing white rats which were fed diets containing chemical agents used in food products or as a sterilizing agent. The amount of gain produced was used as an indication of the toxicity of these chemical agents. It is recognized that the method of measuring toxicity by the amount of gain produced has a limited application.

Neither calcium propionate nor sodium propionate, when fed in quantities up to 3.0 per cent of the diet, decreased the amount of gain.

Zephiran, a disinfectant made up of high molecular alkyldimethylbenzylammonium chlorides, when included in the diet at the rate of 3.0 per cent,

did not cause any ill effect. In fact, the zephiran group made slightly greater gain than the control group; and out of seven pairs of rats finishing the trial, five of the rats receiving zephiran made greater gains than their paired members in the control group. The results of this study indicated that zephiran is not toxic, and since it is a germicide there is a possibility that it could be employed to help control the bacterial flora of the alimentary canal in experiments where that feature is desired.

Sodium benzoate fed at the rate of 1.0 per cent in the diet did not affect the amount of gain. When the rate was increased to 3.0 per cent, definite toxic effects were observed. Numerous investigations have indicated that benzoic acid in the animal organism is detoxified by combining with glycine to form hippuric acid. It has been suggested that the animal organism has a readily available supply of glycine which can be used for the detoxication of benzoic acid. This study indicates that the animal organism has only a limited capacity for detoxifying benzoic acid in the body. The fact that addition of glycine to the sodium benzoate diet increased the amount of gain indicates a decrease in toxicity. The small difference between the gains produced in this group and the control group is considered significant, however, because individual comparisons showed that the control animals made greater gains than the experimental animals in every case.

The animal organism may develop a tolerance for sodium benzoate. Three groups of rats, which had received, respectively, no sodium benzoate, 1.0 per cent sodium benzoate, and 3.0 per cent sodium benzoate plus glycine, were all placed on a diet containing 3.0 per cent sodium benzoate. The group which had not previously received any sodium benzoate made the smallest gain and required the most food per gram of gain. The development of a tolerance for sodium benzoate was indicated by this fact and by the evidence obtained in series II that the toxic effects are expressed during the first 20 days of feeding.

The paired-feeding method of controlling food intake is believed essential to a study of this nature. If the food intakes of the control and experimental animals had not been equal, observed differences in amount of gain could have resulted from the difference in amount of food consumed.

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Biochem. J. 82: 29P, 1962.

The Effect of Phenolic Acids on Cerebral Enzymes

By JOCELYN M. HICKS, I. D. P. WOOTTON and D. S. YOUNG. (*Department of Chemical Pathology, Postgraduate Medical School of London, London, W. 12*)

Many phenolic acids have been identified as abnormal constituents in the plasma of patients with acute renal failure (Hicks, Young & Wootton, unpublished results). Since most of the signs of clinical uraemia can be attributed to depression of cerebral function, the effect of these compounds on the metabolism of the central nervous system has been studied. The following enzymes in rat-brain homogenates have been investigated: glutamic acid decarboxylase, dihydroxyphenylalanine decarboxylase, 5-hydroxytryptophan decarboxylase, 5-nucleotidase, amine oxidase, lactic dehydrogenase, glutamic oxaloacetic and glutamic pyruvic transaminases. The decarboxylases were estimated manometrically while colorimetric or spectrophotometric methods were used for the remaining enzymes. The effect of phenolic acids on the respiration of guinea-pig-brain slices was also investigated.

In any one enzyme system, the molar concentration of the substances tested was the same; this concentration was adjusted so that a considerable inhibition was produced by at least one of the substances. Cinnamic acid and its hydroxylated derivatives were found to inhibit respiration of brain slices to the greatest extent, but phenylpropionic, benzoic and hippuric acids were equally toxic towards glutamic acid decarboxylase; substituted cinnamic acids were less toxic and benzoic acid less toxic still. Dihydroxyphenylalanine decarboxylase was considerably inhibited by 2- and 3-hydroxy derivatives of benzoic, phenylacetic and cinnamic acids; by contrast the 4-hydroxy derivatives were comparatively non-toxic. Aminobenzoic acids were more inhibitory to 5-nucleotidase than any of the other compounds studied.

It is considered that the action of these acids may be important in the abnormality of cerebral function manifested by patients with clinical uraemia.

Australian J. Exptl. Biol Med. Sci. 25(1): 47-56, 1947.

THE RELATION BETWEEN SOME REVERSIBLE REACTIONS OF HAEMOGLOBIN AND THE FORMATION OF GREEN DERIVATIVES.

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(Accepted for publication 8th October, 1946.)

For some years it has been known that, in addition to denaturation by heat and other agents which they suffer in common with other native proteins, haemoglobin and some of its derivatives can undergo reversible reactions characterized by temporary changes in spectrum or, in some cases, in solubility. By some authors these changes have been regarded as evidence of a genuine denaturation of the protein and its subsequent renaturation, or perhaps it should rather be said that the classical definition of denaturation has been expanded to include this relatively recently observed class of phenomena. As an example of the accommodating type of definition now in vogue in this field one may perhaps quote: "Denaturation may be defined as some intra-molecular re-arrangement of the protein giving rise to definite changes in the chemical, physical and biological properties" (Anson, 1938). In quoting this definition, Neurath, Cooper and Erickson (1942) carefully add a footnote of their own categorically excluding from "intra-molecular re-arrangement" "the reversible splitting of native proteins into smaller units, or reactions with groups on the surface of the molecules which leave their internal structure intact." If "intact" is intended to mean "unchanged," it would seem that the footnote excludes very little since, e.g. it is almost certain that the conversion of methaemoglobin to oxyhaemoglobin profoundly alters the internal structure of the globin molecule, just as it does some of its physical and chemical properties.

To include in one category these reversible reactions almost peculiar to the blood pigment and those occurring when, say, egg albumin is dropped into alcohol may perhaps be not unjustly likened to the use of one phrase to include both a gentle squeeze of a tennis ball between the fingers and the turning of the said ball, after suitable dissection, inside out. Nor does it seem easy to align the fact that denaturation, as usually understood, consists in the rupture of a large number of weak bonds in a native protein of almost unique constitution, thus transforming it into one of exceedingly random structure (Mirksy and Pauling, 1936) with the extraordinary ease with which some reactions termed denaturation have been reversed. There is another class of reactions of the blood pigment by means of which are produced green pigments possessing in the ferrous state a strong absorption band in the red region of the spectrum. Most of these pigments have not yet been obtained in a pure state, nor have their constitutions and relationships been fully established. The common feature in the reactions appears to be the action of an oxidizing agent on one of the ferrous derivatives of the blood pigment. A comparative study of the reversible changes that the blood pigment can endure and of the preparation of green pigments has extended the list of each type of reaction and suggests that they may be more closely connected than would at first seem likely.

[1]This work was aided by a grant from the Australian National Health and Medical Research Council.

Whatever one's view of protein denaturation, it is helpful to distinguish accurately between different types of reactions of proteins; the practice in this paper follows that of previous ones in this series of reserving the term denaturation for examples of those reactions whose resultants come within the classical definition applicable to denatured proteins from any source: *i.e.* that they can be repeatedly precipitated at their iso-electric points but are soluble away from them. As a simple term for reversible reactions of the blood pigment whose resultants are readily convertible to it the word "perturbation" will be used and the word "restoration" for the reconversion of such a resultant to a protein soluble at its iso-electric point and containing unaltered haem or haematin. As yet the occurrence of analogous reactions with other native proteins is, perhaps, a matter for conjecture rather than for affirmation.

EXPERIMENTAL.

The experiments to be described fall into three main groups: 1, a study of the effects on haemoglobin and methaemoglobin of certain reagents known to denature oxyhaemoglobin; 2, the preparation from haemoglobin of various green pigments with absorption bands in the red region of the spectrum; and 3, the separation from the protein of the prosthetic groups of a few of these green pigments.

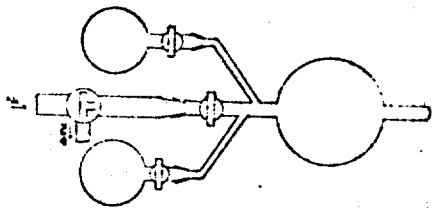

Fig. 1.

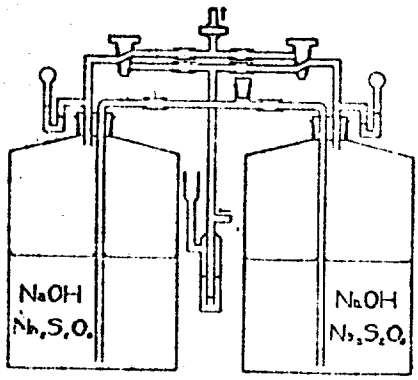

Fig. 2.

The results of many diverse experiments have demonstrated that reducing agents, particularly sodium hyposulphite, have an action on oxyhaemoglobin very different from that of an indifferent gas or a reduction in the gaseous pressure in desoxygenating it. Accordingly the studies on haemoglobin were again pursued in an apparatus similar to that previously used (Holden, 1937) for this purpose. The rubber tubing was replaced by ground joints and the screw clips by taps. The apparatus was diminished to a total volume of 150 ml. and to the main bulb was added a tube in which observations on a film of liquid could be made with a Hartridge spectrometer supported vertically. (Fig. 1). The apparatus was mounted on a board that was rotated at about 100 r.p.m. by a motor. The only change in the apparatus for supplying nitrogen freed from oxygen was a corresponding replacement, as far as possible, of rubber by glass and clips by taps. (Fig. 2). The solutions were placed in the three flasks with capillary pipettes; the technique was otherwise unchanged. To ensure that experiments on methaemoglobin should not be vitiated by undetected leakage of air a small amount of oxyhaemoglobin was added to the harmless restorative solution. Where it was desired to saturate the haemoglobin solution with a reagent a moderate excess of the latter in the solid state was placed in the largest flask and the haemoglobin occupied the small one usually reserved for the perturbator.

As solutions of haemoglobin and oxyhaemoglobin washed and laked erythrocytes were used. Methaemoglobin was prepared by autoxidation of oxyhaemoglobin at 37° C. and pH 5·3. Its freedom from undesirable reagents such as traces of potassium ferricyanide outweighed the disadvantage of its including a small percentage of native protein joined to an altered prosthetic group. Experiments showed that crystallized horse cyanmethaemoglobin prepared by autoxidation had a maximum visible extinction coefficient 5–7 p.c. less than that prepared

by the oxidation of oxyhaemoglobin with potassium ferricyanide. It seems, however, very difficult to effect a complete removal of the latter. The methaemoglobin was converted as required to cyanmethaemoglobin with the minimal quantity of potassium cyanide neutralized with acetic acid. The degree of restoration was judged sufficiently closely for present purposes by a comparison of the spectrum of the restored and reduced protein solution with that of a double wedge cell, one compartment of which contained a solution of haemoglobin and the other an equivalent solution of denatured globin haemochromogen. Only where the degree of denaturation was very small was it necessary to separate the denatured protein and determine it separately.

TABLE 1.

A. Substances which denature oxyhaemoglobin but which have little irreversible effect on haemoglobin.

Ammonia.
Ethanol.
Potassium iodide.
Sodium benzoate.
Sodium salicylate.
Sodium benzene sulphonate.

B. Substances which denature any native derivative of haemoglobin.

Phenol.
Pyridine.
Formaldehyde.
Ammonium thiocyanate.
Potassium thiocyanate.

C. Substances which do not denature oxyhaemoglobin but which combine with haemoglobin and promote the formation of green pigments.

Potassium cyanide.

TABLE 2.

The effect of acid on the derivatives of haemoglobin.

Sulphuric acid was used because it is not volatile. The period of action was 5 minutes.

A. Derivatives of horse haemoglobin.

Derivative.	Concn. gm./litre.	Vol. ml.	Gas.	Concn. of acid, N.	Vol. of acid, ml.	pH reached.	Temp. C.	Alkaline restorer.	Concn. of alkali, N.	Vol. of alkali, ml.	p.c. restored.	Remarks.
Hb.	77·5	2·5	N_2	0·5	1·3	1·65	0°	Na_2CO_3	1	2·5	90	
Hb.	81	2·0	N_2	0·5	2·0	1·3	0°	Na_2CO_3	1	4·0	85	
Hb.	77·5	2·5	N_2	0·5	1·3	1·65	20°	Na_2CO_3	1	2·5	85	
Hb.	81	2·0	N_2	0·5	2·0	1·3	17°	Na_2CO_3	1	4·0	80	
HbO_2.	77·5	5·0	Air	0·5	2·6	1·65	20°	Na_2CO_3	1	5	Trace.	
Methb.	107	1·0	N_2	0·05	2·0	3·84	0°	Na_2CO_3	0·1	5	100	Minimal amount of acid to convert to acid haematin.
Methb.	107	1·0	N_2	0·05	2·0	3·84	16°	Na_2CO_3	0·1	5	90	
Methb.	107	1·0	Air	0·05	2·0	3·84	0°	NH_3	0·1	2	75	
Methb.	107	1·0	Air	0·05	2·0	3·84	16°	NH_3	0·1	2	50	
CN.Methb.	30	2·0	Air	0·05	3·0	3·6	0°	NH_3	0·1	5	100	No acid haematin formed.
CN.Methb.	30	2·0	Air	0·05	4·0	2·33	0°	NH_3	0·1	5	Little.	

B. Derivatives of human haemoglobin.

Derivative.	Concn. gm./litre.	Vol. ml.	Gas.	Concn. of acid, N.	Vol. of acid, ml.	pH reached.	Temp. C.	Alkaline restorer.	Concn. of alkali, N.	Vol. of alkali, ml.	p.c. restored.	Remarks.
Hb.	68	2·0	N_2	0·05	4·0	3·34	0°	Na_2CO_3	0·1	10	100	Note low acidity sufficient to convert to acid haem.
Hb.	68	2·0	N_2	0·05	4·0	3·31	16°	Na_2CO_3	0·1	10	100	
HbO_2.	69	5·0	Air	0·5	1·0	3·3	22°	Na_2CO_3	1	2·5	Little.	
Methb.	137	1·0	N_2	0·05	2·5	4·6	0°	Na_2CO_3	0·1	5·0	100	Note low acidity sufficient to convert to acid haematin.
Methb.	137	1·0	N_2	0·05	2·5	4·6	16°	Na_2CO_3	0·1	5·0	90	
Methb.	137	1·0	Air	0·05	2·5	4·6	0°	NH_3	0·1	3·0	90	
Methb.	137	1·0	Air	0·05	2·5	4·6	16°	NH_3	0·1	3·0	85	
CN.Methb.	180	0·5	Air	0·5	0·8	1·8	15°	NH_3	1	1·0	50	Minimum acid to convert to acid haematin.

The effects of perturbators differ with the temperature of the reaction, the derivative of the blood pigment selected and the species whence it is obtained. For two perturbators, hydrogen ions and ethanol, a comparative study was mode of four derivatives: haemoglobin, oxyhaemoglobin, methaemoglobin and cyanmethaemoglobin of the horse and of man. The last two derivatives were studied both in the presence and in the absence of oxygen. To allow time for a complete admixture of the perturbator with the pigment derivative and a spectrometric observation with the Hartridge while restricting extraneous effects due to a prolonged exposure to the action of the perturbator, e.g. hydrolysis, a standard time was adopted of five minutes between the addition of the perturbator and that of the restorer. The details of quantities used and the results are in Tables 1–5. Potassium cyanide occupies an apparently unique position. A drop of 15 p.c. horse oxyhaemoglobin in 1 ml. of 30 p.c. potassium cyanide was unaltered. On reduction the spectrum of cyanhaemochromogen with the weak α band was seen which on dilution changed after several minutes to that of haemoglobin. The structure of the first substance cannot be at present defined. It has been established by magnetic measurements that the substance with a very different spectrum formed temporarily when cyanmethaemoglobin is reduced is the true cyanhaemoglobin analogous to carbon monoxide haemoglobin. Unlike other chemicals that were used for perturbation the temporary precipitation near the neutral point usually so characteristic was not observed when potassium cyanide acted on haemoglobin or oxyhaemoglobin. There was only a little salting-out at very high concentrations.

TABLE 3.

The effect of precipitation with ethanol in neutral solution on derivatives of haemoglobin.

Unless otherwise stated the restoring agent was 10 ml. water.
The restoring agent was added 5 min. after the ethanol.

Derivative.	Concn. gm./litre.	Vol. ml.	Gas.	Vol. of ethanol, ml.	Temp. C.	p.c. restored.	Remarks.
A. Derivatives of horse haemoglobin.							
Hb.	150	1·0	N_2	2·0	0°	90	
Hb.	150	1·0	N_2	2·0	20°	80	
Methb.	107	1·0	N_2	1·5	0°	90	Ppt. insol. in H_2O. Restored with 0·2M Na_2CO_3.
Methb.	107	1·0	Air	1·5	0°	75	Ppt. insol. in H_2O. Restored with 0·2M Na_2CO_3.
Methb.	107	1·0	N_2	1·4	19°	80	Ppt. insol. in H_2O. Restored with 0·1M Na_2CO_3 and $Na_2S_2O_4$.
Methb.	107	1·0	Air	1·0	16°	70	Ppt. insol. in H_2O. Restored with 0·1M NH_3 and $Na_2S_2O_4$.
CN.Methb.	106	1·0	N_2	1·2	0°	100	
CN.Methb.	106	1·0	Air	1·2	0°	95	
CN.Methb.	106	1·0	N_2	1·2	18°	75	Ppt. insol. in H_2O. Restored with 0·1M NH_3.
CN.Methb.	106	1·0	Air	1·2	18°	65	Ppt. insol. in H_2O. Restored with 0·1M NH_3 and $Na_2S_2O_4$.
B. Derivatives of human haemoglobin.							
Hb.	136	1·0	N_2	2·0	0°	95	
Hb.	136	1·0	N_2	2·0	17°	80	
Methb.	137	1·0	N_2	1·1	0°	90	Ppts. insol in H_2O. Restored with 0·1M NH_3 and $Na_2S_2O_4$.
Methb.	137	1·0	N_2	1·1	17°	40	
Methb.	137	1·0	Air	1·0	0°	40	
Methb.	137	1·0	Air	1·0	16°	0	Ppt. insol. in NH_3. Dissolved with dil. NaOH.
CN.Methb.	180	1·0	N_2	1·5	0°	65	
CN.Methb.	180	1·0	N_2	1·5	17°	50	
CN.Methb.	180	1·0	Air	1·0	0°	40	
CN.Methb.	180	1·0	Air	1·0	15°	40	

The human derivatives are in general more readily denatured than the horse derivatives.

From these experiments the following conclusions may be drawn. There are well-marked differences of species; neither horse nor human blood pigment derivatives are as resistant to denaturation as those of the ox previously investigated. Human derivatives are remarkably easily perturbed by acid in comparison with those of the horse and ox. No record has come to my notice of the haemoglobin of any common animal being converted to acid haem at so low an

acidity as pH 3·3. Human methaemoglobin is even more remarkable, being perturbed and partially denatured at pH 4·6 within five minutes. Experiments on the effect of acid on cyanmethaemoglobin showed that it could withstand a pH that denatured much of the corresponding methaemoglobin. There is, however, no spectral change at that pH and so the results have not been tabulated. To dissociate the hydrogen cyanide from the haematin required an acidity of about pH 2·3 and at that pH denaturation was very extensive. An important point is that methaemoglobin is much more easily denatured (as well as more easily perturbed) than haemoglobin. The difference in stability towards acid between acid haematin derived from methaemoglobin and acid haem from haemoglobin points either to haem being able to stabilize the protein in a way impossible to haematin or to some essential differences within the protein molecules existing before the addition of the acid. Though direct evidence of such a difference may not be readily forthcoming it must be remembered that all the methods of producing methaemoglobin involve a powerful oxidative action, whether the ferricyanide, ferric sulphate or autoxidation method be used. Under such a stress some modification of the protein may well occur.

TABLE 4.

The perturbation of horse haemoglobin by reagents other than acids or ethanol.

The perturbations lasted for five minutes.

Volume of haemoglobin solution. ml.	Conc. of haemoglobin solution. gm./litre.	Temp. C.	Perturbator.	p.c. perturbed.	Restorer.	p.c. restored of that perturbed.
1	72	19°	3 ml. conc. NH_3	100	11 ml. 16 p.c. H_3PO_4	95
1	72	19°	3 ml. 30 p.c. KCN	100	10 ml. 10 p.c. NH_4Cl	95
1	150	0°	3 ml. 30 p.c. KCN	100	10 ml. 5 p.c. H_3PO_4	100
1	150	16°	excess solid sodium benzoate	100	10 ml. H_2O	100
1	150	17°	excess solid KI	20	10 ml. H_2O	100
1	151	20°	excess solid sodium benzene sulphonate	25	10 ml. H_2O	100

The difference between methaemoglobin and other derivatives is shown in several ways. At laboratory temperatures the denaturation in saturated urea solution of horse or human haemoglobin or oxyhaemoglobin is very slow. The two cyanmethaemoglobins in neutral solution are unaltered by saturation with urea. No precipitates are formed on dilution and on reduction haemoglobin is, after the first few minutes, the sole product. The cyanmethaemoglobins were perturbed by saturation of their solutions with potassium iodide or sodium benzoate and precipitated on dilution with water. On dissolving the precipitates with dilute ammonia and treating with sodium hyposulphite it was found that scarcely any had been denatured. The methaemoglobins, however, were perturbed by urea, precipitated on dilution of their solutions with water and a test similar to the last showed that 20 p.c. of each protein was denatured. In like manner potassium iodide and sodium benzoate denatured half of each methaemoglobin.

Among other reagents used to perturb or denature haemoglobin were the following: pyridine, phenol, formalin, sodium salicylate, sodium benzoate, potassium iodide, potassium thiocyanate, ammonia and potassium cyanide. The first three have a direct denaturant action and little, if any, restoration can be observed. Sodium salicylate, sodium benzoate, concentrated ammonia, potassium cyanide and potassium iodide changed the haemoglobin into a substance with a spectrum resembling that of haemochromogen, in the case of potassium cyanide that of cyanhaemochromogen. Potassium iodide only perturbed part of the haemoglobin. In all these cases the appropriate restorer (shown in Table 4) completely restored the pigment to haemoglobin. Potassium thiocyanate both oxidized the iron of haemoglobin and denatured it. No restoration could be observed. The wave-lengths of the α bands of the haemochromogens seen on perturbation are given in Table 5. They differ from that of denatured globin haemochromogen.

TABLE 5.

Wave-lengths in mμ of the α bands of haemochromogen-like compounds resulting from the treatment of horse haemoglobin with the reagents indicated.

Reagent	mμ	Reagent	mμ
Sodium benzoate	561	Ammonia	559
Sodium salicylate	560	Potassium iodide	561
Sodium benzene sulphonate	560	Potassium cyanide	567

All the above were restored to haemoglobin. Pyridine produced a haemochromogen with a band at 557. This substance was completely denatured. The spectrum observed with potassium cyanide resembled that of cyanhaemochromogen rather than that of reduced cyanmethaemoglobin, which has a typical haemochromogen spectrum with an intense α band at 565 mμ.

It is known that if a solution of haemoglobin, perturbed by acid, be neutralized suddenly with sodium carbonate or better with sodium phosphate, an insoluble pigment whose spectrum is that of a haemochromogen is formed. The precipitate is denatured by oxygen, but on careful solution in its absence with the minimum of acid or alkali it is restored to haemoglobin. Apart from the denaturation by oxygen a similar precipitate was seen when methaemoglobin was similarly perturbed. In the course of the experiments of this paper a like effect was observed when methaemoglobin but not when haemoglobin or cyanmethaemoglobin was precipitated with alcohol. It frequently happened that a precipitate which was quite insoluble in water was found when treated with dilute ammonia or ammonia and sodium hyposulphite to be largely composed of restorable protein. When haemoglobin or cyanmethaemoglobin was precipitated with alcohol the native protein could be dissolved away readily in water leaving, in the latter case, some insoluble denatured protein behind. A ready distinction of these complexes of perturbed and denatured blood pigment derivatives from pure denatured derivatives is the ease with which the former break up in dilute ammonia, the native protein going into solution. A precipitate composed solely or mainly of denatured material requires dilute sodium hydroxide to dissolve it. For any derivative the amount of restoration observed decreased with increase of temperature. Though less strongly than with haemoglobin a definite increase in denaturation due to oxygen was observed when methaemoglobin or cyanmethaemoglobin were treated with acid or alcohol. Methaemoglobin is both less soluble than haemoglobin or cyanmethaemoglobin in alcohol-water mixtures and is more readily denatured by alcohol.

TABLE 6.

Wave-lengths in mμ of the bands in the red region of carbon monoxide compounds of green pigment formed from horse haemoglobin in the presence of various promoters.

Promoter.	Formed with $Na_2S_2O_4$ and O_2	Formed with $Na_2S_2O_4$ and H_2O_2
Pyridine	623	627
Phenol	624	628
Ethanol	625	629
Hydrazine (no other reducer)	618	624
Sodium benzoate	621	628
Sodium salicylate	625	628
Sodium benzene sulphonate	624	629
Potassium iodide	620	628
Potassium cyanide	620	631
Formaldehyde	625	615 (no $Na_2S_2O_4$)

The compound formed using hydrazine and oxygen probably contained denatured material.

The second group of experiments consisted in the preparation of green pigments by the repeated desoxygenation of horse oxyhaemoglobin with sodium hyposulphite in the presence of various chemicals (termed "promoters") and its re-oxygenation with atmospheric oxygen. They were performed in test-tubes, the tubes being rotated at a small angle with the horizontal to oxygenate the liquid without undue frothing. In parallel experiments the desoxygenated pigment was at once treated with a few drops of 1 p.c. hydrogen peroxide. In some cases the preliminary desoxygenation was found unnecessary and the mixture of oxyhaemoglobin and promoter was treated directly with hydrogen peroxide. The first chemicals tested as promoters were those in Tables 4 and 5 used in relatively low concentrations. Afterwards a larger range of substances was similarly tested. In nearly every case it was found that two pigments were formed according to whether oxygen or hydrogen peroxide were employed. The absorption

band of the carbon monoxide derivative prepared with oxygen had in general a wave-length a few mμ shorter than that with hydrogen peroxide. The wave-lengths of the bands varied somewhat irregularly for different promoters and even for different specimens prepared using the same promoter. It would appear probable that each of the two green pigments formed through the agency of a given promoter was contaminated to a variable degree by the other. The details are in Table 6. The difference in the two series is probably due to modifications in the vinyl side-chains of the prosthetic group brought about by the prolonged action of sodium hyposulphite and oxygen.

A group of substances that have well-marked disintegrative effects on the blood pigment are aromatic hydroxyl and quinone derivatives. Thus phenol denatures haemoglobin but in lower concentration acts as a promoter of green pigment formation. If α naphthol in a weakly alkaline solution be added to oxyhaemoglobin a portion of the pigment is rapidly converted to alkaline haematin and another part transformed to a green pigment with a band like those obtained through the action of hydrogen peroxide. Naphthoquinone rapidly converts oxyhaemoglobin to a mixture of denatured globin parahaematin and a similar green pigment. In neutral solution quinol turns oxyhaemoglobin to methaemoglobin. In weakly alkaline solution its action is similar to that of α naphthol. In these cases the one substance appears both to act as a promoter and, by interaction with the oxygen of the blood pigment, as a furnisher of the necessary hydrogen peroxide.

TABLE 7.

The separation of the prosthetic group from some green pigments and the wave-lengths of the bands of its carbon monoxide derivative. Horse oxyhaemoglobin, sodium hyposulphite and oxygen were used.

		Wave-length, mμ of CO compounds of free group.			Wave-length, mμ of CO compounds of free group.
Phenol	0·3 g.		Pyridine	1 ml.	
Oxyhaemoglobin 16 p.c.	10 ml.	614	Oxyhaemoglobin 13 p.c.	25 ml.	616
Na_2CO_3 10 p.c.	4 ml.		$N.NH_3$	10 ml.	
H_2O	20 ml.		H_2O	45 ml.	
α naphthol	0·2 g.		Potassium iodide	5 g.	
Oxyhaemoglobin 13 p.c.	25 ml.	612	Oxyhaemoglobin 2·7 p.c.	50 ml.	616
$N.NH_3$	20 ml.		$N.NH_3$	20 ml.	
H_2O	40 ml.		H_2O	50 ml.	
Sodium salicylate	12 g.		Hydrazine hydrochloride	0·5 g.	
Oxyhaemoglobin 13 p.c.	25 ml.	626	Oxyhaemoglobin 15 p.c.	25 ml.	614
$N.NH_3$	10 ml.		Na_2CO_3 10 p.c.	4 ml.	
H_2O	45 ml.		No sodium hyposulphite		

The third group of experiments involved the separation from the protein of the prosthetic groups of some of the green derivatives. The methods of their preparation in suitable amount and of the separation of the prosthetic group were essentially those already described for the preparation of cruoratin (Holden, 1945), starting however from oxyhaemoglobin and using a concentration of the promoter found suitable in test-tube experiments. The details and the wave-lengths of the bands of the CO compounds of the prosthetic groups are in Table 7. The yields were always small compared with that obtained from cruoralbin. This appears to be due partly to a further oxidative destruction of the green pigment whose concentration appears to reach a maximum and then to diminish. Other reactions apparently also occur. Bile pigments were always found. In addition to its function as a promoter, cyanide appears to impart a certain small degree of stability to the cruoralbin produced under its influence, impeding its further destruction. Incidentally bile pigments have not been found in solutions of cruoralbin.

The tint of the solution of the green pigments formed in some experiments showed that much of the haem was being broken down in a direction different from that leading either to choleglobin or to cruoralbin. The solutions often developed a slatey-bluish colour—later turning almost black. One of the most striking examples of this was when a small amount of copper sulphate in normal ammonia was used as a promoter. The pigment was extracted from a large preparation with amyl alcohol and acetic acid as usual, the acid removed by washing and the pigments taken into dilute sodium hydroxide. An example may be quoted of another means for the oxidative destruction of haemoglobin, the use of hydrazine. To a 10 p.c. solution of horse oxyhaemoglobin, containing the stroma, was added some hydrazine sulphate dissolved in excess of ammonia. The mixture was placed in a shallow dish at 0° C. for several days. When most of the blood pigment appeared to have been destroyed the pigments were extracted with amyl alcohol and acetic acid. After being removed thence into aqueous ammonia it was chromatographed over barium oxalate. The carbon monoxide compound so obtained had a band of 614 mμ, near to that of cruoraem carbon monoxide, so that its vinyl side chains had presumably been attacked.

DISCUSSION.

Any attempt to formulate for these experiments a unifying mechanism or to derive from them some information about the constitution of the blood pigment encounters a serious difficulty at the outset. So much of the constitution of the blood pigment and its immediate derivatives has been rigidly established on the basis of titration curves, determinations of magnetic susceptibility, measurements of reaction velocities at varying temperatures and other physical measurements, that purely chemical facts have to be fitted into this framework as best they can. The prosthetic group, whether ferric or ferrous, is linked to the globin by two iminazolyl groups of histidine molecules placed just too far apart for co-ordination. Any substance, except a simple ion, which combines with the blood pigment or a derivative does so by uniting with the iron atom of the prosthetic group. All other possibilities have apparently been excluded.

Though not all compounds of blood pigment derivatives have covalent linkages around the iron atom the native protein can only be so linked to the prosthetic group when another smaller molecule such as carbon monoxide is also covalently linked to the iron atom of the prosthetic group. The effect of ammonia in diminishing the magnetic susceptibility of haemoglobin and the transitory spectrum seen during the reduction of cyanmethaemogloblin have been pronounced to be proofs of the formation of ammonia haemoglobin and cyanhaemoglobin analogous to carbon monoxide haemoglobin. The spectrum of ammonia haemoglobin was apparently not observed, but it is now seen to be that of a haemochromogen. It is of interest to note that ammonia haemoglobin, unlike carbon monoxide haemoglobin, is denatured by oxygen. That ammonia has a reversible effect on the globin, apart from the formation of ammonia haemoglobin, is shown by the fact that on the addition to it of the first few drops of dilute phosphoric acid, while the solution was still alkaline, most of the protein was precipitated but redissolved on the addition of the rest of the acid which made the solution nearly neutral. The increase in the magnetic susceptibility of alkaline methaemoglobin in the presence of a large amount of ethanol demonstrated the combination of these substances. The alteration in the spectrum of alkaline methaemoglobin (not of haemoglobin as is so often stated in error) in the presence of sodium salicylate proved the denaturation of the former (Anson and Mirsky, 1934). Curiously enough the change of spectrum of methaemoglobin in the presence of sodium fluoride proves the existence of a compound between them and not, as in the preceding instance, of a denaturation though it is equally reversible.

It may well be argued from one or other of the preceding examples that the haemochromogen spectra observed when haemoglobin is in the presence of potassium iodide, sodium salicylate, sodium benzene sulphonate, ethanol, etc., are proof that these substances combine with haemoglobin as does carbon monoxide, or, perhaps, as pyridine does with myoglobin, but not with haemoglobin (Kiese and Kaeske, 1942). It may be here mentioned that if at pH 9 a solution of alkaline horse methaemoglobin be treated with a little dilute formalin solution, also just previously adjusted to pH 9, the spectrum changes at once to that of methaemoglobin, which is then slowly converted to that of alkaline haematin with but little denatured globin parahaematin. On reduction it yields mostly haem with a little denatured globin haemochromogen. By current reasoning this would indicate the formation of a temporary compound of formaldehyde and methaemoglobin with a spectrum resembling that of the latter. Holden and Freeman (1929) showed that formaldehyde greatly diminishes the ability of denatured globin to form parahaematin or haemochromogen. In contrast they

found that formaldehyde has no effect on the formation of parahaematins or haemochromogens by simple iminazole compounds. This has been re-examined and confirmed. Thus each molecule of haem combines with two iminazole groups of native globin while in denatured globin haemochromogen at most with only one. The nature of the second group involved is unknown—apparently it is a primary or secondary amine. If a little phenol be added to a solution of haemin dissolved in dilute sodium hydroxide solution the spectrum changes at once to that of acid haematin though the solution is still alkaline. Whatever be the explanation, one hestitates to add phenols to the already long list of compounds that combine with haematin.

Unlike other compounds under discussion, carbon monoxide haemoglobin has a spectrum resembling that of carbon monoxide haem and of carbon monoxide denatured globin haemochromogen instead of having as might be expected a haemochromogen spectrum. Among the simpler compounds of haem there is but one known which shows a true haemochromogen spectrum without both the iron valencies external to the porphyrin ring being attached to suitable nitrogen atoms. Many years ago Anson and Mirsky (1928), in a paper whose importance seems to have been overlooked, showed that haem combines with hydrogen cyanide in equi-molecular proportions to form a substance showing the visible spectrum typical of a haemochromogen, the sixth co-ordination place around the iron atom being filled with a non-nitrogenous substance or group (water or OH′). It has also long been known (see, e.g. Hill, R., 1926, Pauling and Coryell, 1936) that for all other simpler bases such as pyridine or nicotine two molecules of base are required per molecule of haem for the formation of a haemochromogen. The work of Anson and Mirsky permits of the haemochromogen spectra of perturbed haemoglobin being interpreted as due to the co-ordination of the iron atom of the haem with one nitrogen atom of the globin and a non-nitrogenous substance such as are many of the perturbators. It does not much assist in finding a common factor in so assorted a group of substances.

In August, 1946, Mr. J. P. Callaghan communicated to the Australian and New Zealand Association for the Advancement of Science the results of a series of determinations with a Hardy recording spectrophotometer of the absorption spectra of solutions of haem containing cyanide ions. He confirmed the observation of Anson and Mirsky (1928) of the existence of two compounds of haem with cyanide. He showed that the four-banded spectrum seen under certain conditions is due to a mixture of the two compounds. On the assumption that in his experiments all the haem in the "monocyanide" compound is combined with cyanide the maximum Σ is 15,100. This assumption has not yet been proved, but, if correct, the Σ is only about half that of pyridine protohaemochromogen though the spectra are otherwise similar.

Denaturation has been shown to be a change, involving the rupture of many weak hydrogen bonds, from an almost unique to an exceedingly random type of structure. If the temporary conversion of haemoglobin to compounds with a haemochromogen spectrum is a temporary denaturation rather than a formation with divers reagents of a series of compounds all possessing this structure and spectrum, it is a little difficult to reconcile the extensive changes in the structure of the globin molecule that are implied with the ease with which these peculiar reactions can be reversed. A separate classification is perhaps not altogether unjustified.

While carbon monoxide haemoglobin and nitric oxide haemoglobin are essentially unchanged in structure by molecular oxygen, a much longer list is oxidatively disintegrated by this gas with the formation of various types of break-down products. The list can undoubtedly be greatly [illegible]

to whom adequate stocks of chemicals are available. It is not easy to see, on current theories, wherein lies the peculiar virtue of carbon monoxide as compared, with say, potassium iodide or cyanide in preventing the disintegration by oxygen of its compound with haemoglobin. The day when the chemist might attempt to describe, if not elucidate, the phenomenon with the help of separate constitutional formulae for these substances differing so in their reactions appears to be over.

It can be seen that sodium hyposulphite, and this holds for most reducing agents, is not so innocent an absorbent of oxygen as it might seem. Since oxyhaemoglobin is dissociable, a reducing agent would be unnecessary for the formation of green pigments if its sole function were to promote the dissociation by reacting with the oxygen dissolved in the solution.

It is attractive to imagine that a perturbing agent forms a dissociable compound with haemoglobin. In view of the probable number of such compounds, of the apparently entirely unrelated natures of the perturbing agents and of the almost uniform haemochromogen spectra of the compounds formed, it becomes increasingly difficult to assign all the responsibility to the iron atom. There, however, it must rest while the existing structure of haemoglobin is maintained.

That the group of reagents under survey can under suitable conditions perturb haemoglobin, assist oxygen to denature oxyhaemoglobin or promote the formation of green pigments would indicate some relation between these reactions. A haemoglobin perturbed is usually too readily denatured by oxygen for it to be, except in a few cases, a very likely intermediate in the formation of a green pigment. Since a lower concentration of a perturbator than is required for a visible degree of perturbation is adequate for the promotion of green pigment formation, one is tempted to assume an intermediate step whereby a little of the haemoglobin is modified in such a way that the molecular oxygen neither unites with the iron atom nor so reacts as to denature the protein, but takes a third course.

SUMMARY.

A number of substances which effect the denaturation of oxyhaemoglobin convert haemoglobin to substances with a haemochromogen spectrum. In the absence of oxygen the phenomenon can be reversed.

The same substances are among those which promote the formation of green pigments through the oxidative disintegration of haemoglobin.

The list includes ammonia, ethanol, potassium iodide, sodium benzoate, and other aromatic compounds.

The different effects of acid and of ethanol on four derivatives of the blood pigments of horse and of man are described.

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Arch. Biochem. 22: 345-352, 1949.

Changes in the Prothrombin Time Induced by Methylxanthines. Role of the Plasma Cofactor of Thromboplastin

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Received January 28, 1949

Introduction

In 1943, Topelberg and Honorato (1) demonstrated that caffeine can shorten the prothrombin time of chickens maintained on a vitamin K-deficient ration, but only temporarily and with a weaker action than vitamin K. Honorato, Ivanovic and Palma (2) found a similar action on the prothrombin time of rats on a low choline diet. Field *et al.* (3) subsequently studied the action of large doses of caffeine, theobromine and theophylline on dogs, rabbits and rats, using the one-stage method of Quick (4), but diluting the plasma to 12.5% with saline, and obtained a shortening of the prothrombin time for many days after these drugs were administered. Link and coworkers (3a) also indicated that the methyl xanthines augment the fibrinogen level above the normal ranges. Quick (5) was unable to repeat the latter experiments, but he diluted with fresh human plasma treated with $Al(OH)_3$. Scherf and Schlachman (6), working with plasmas diluted to 12.5% with saline, found that caffeine shortens the prothrombin time in man, but Blood and Patterson (6a) reported that aminophyllin had no significant effect on the prothrombin time in man. On the other hand, Deutsch and Gerarde (7) believe that the shortening of the prothrombin time found by Link's group is produced by the fibrinogen exclusively.

We have studied the same problem, particularly with respect to the role of the thromboplastin cofactor, the principle necessary for the conversion of prothrombin into thrombin, with thromboplastin, recently described by the author (8). This factor is adsorbed by $Al(OH)_3$ at a concentration of 0.2 *M* but is not adsorbed when the $Al(OH)_3$ is less concentrated than 0.05 *M*. Furthermore, any preparation of fibrinogen from rabbit or bovine will carry it as an impurity if special precautions are not taken. Thus, any dilution of plasma with human plasma treated with an insufficient amount of $Al(OH)_3$, or with fibrinogen without special treatment, will contain this thromboplastin cofactor (PCT).

More or less simultaneously, Owren (9) described a new factor, the factor V, and Seegers' group (10) the AC-globulin, both resembling PCT action. Nevertheless, we have not analyzed the PCT and thus we are not able to identify it with, or differentiate it from, these factors.

Experimental

Techniques

Caffeine (citrate), theobromine and theophylline were given to rabbits by stomach tube in single doses of 100 mg./kg. body weight. Sodium benzoate was used at the same dose level, also, as a control, because theobromine was dissolved in water with it.

To determine the prothrombin time, the one-stage method of Quick (4) was used with undiluted and diluted plasma. The thromboplastin agent used was dried rabbit brain. Dilutions of 20, 10, and 5% were made with distilled water and human and rabbit fresh plasmas treated with 0.01 *M*, 0.1 *M* and 0.2 *M* $Al(OH)_3$ (used as diluents).

The influence of fibrinogen on the prothrombin time was studied by making dilutions of plasma with human, rabbit, or bovine fibrinogen. The human and rabbit fibrinogen were prepared by treatment of plasma with sufficient NaCl to obtain 50% saturation. Bovine fibrinogen was prepared by the technique of Seegers (11).

TABLE I

Effect of 100 mg./kg. Body Weight of Caffeine, Theobromine, Theophylline and Sodium Benzoate in a Single Dose on the Prothrombin Times of Rabbits, Using 10% Diluted Plasmas

Rabbit	Substance used	Prothrombin time (sec.)						
		Before	After 1 day	2 days	3 days	4 days	5 days	7 days
1	Caffeine		14	8	10	9	—	—
2			14	8.5	11	11	10	15
3			15	10	11.5	10.5	11	15
4			16	10	9	11	11	12
Average		20±2	14.7±0.4	9.1±0.5	10.3±0.5	10.3±0.4	10.6±0.3	14±1
5	Theobromine		14	12	13	12	14	17
6			11	12	10	12	13.5	16
7			12	11	13	11	—	—
8			18	9	11	11	12	14.5
Average		20±2	13.7±3.7	11±1.2	11.7±3.9	11.5±0.5	13±1.2	15.8±1
9	Theophylline		14	10	8.5	9	14.5	
10			10	10	9	—	—	
11			17	9	13	15	17	
12			16	8	11	14	—	
Average		20±2	14±1.5	9.2±0.4	10.3±1.0	12.6±1.6	15.7±1.2	
13	Sodium benzoate	20±2	21	9	11	11	12	14
14 to 27	None	20±2						

Results

Effect of Caffeine, Theobromine, Theophylline and Sodium Benzoate on the Prothrombin Time of Rabbits

The prothrombin time of undiluted rabbit plasma, with a value of 6 sec., was not changed by the administration of any of these compounds. Only thromboplastin, which gave 12 sec. with fresh human plasma, was used. The results are recorded in Tables I and II. The results obtained with 20% diluted plasmas gave no significant changes in comparison with untreated animals.

TABLE II

Effect of 100 mg./kg. Body Weight of Caffeine, Theobromine, Theophylline and Sodium Benzoate in a Single Dose on the Prothrombin Times of Rabbits Using 5% Diluted Plasmas

Rabbit	Substance used	Prothrombin time (sec.)						
		Before	After 1 day	2 days	3 days	4 days	5 days	7 days
1	Caffeine		25	14	30	30		
2			22	15	22	28	34	27
3			24	20	21	32	32	39
4			31	18	21	32	31	30
Average		36±1.7	25.5±1.9	16.7±1.3	23.5±2.1	30.5±1	32.3±1.1	32.5±5.1
5	Theobromine		30	24	30	27	29	39
6			17	20	20	22	27	37
7			23	23	29	22		
8			32	17	20	23	23	29
Average		36±1.7	25.5±3.4	21±1.4	24.7±2	23.5±1	26.3±1	35±3
9	Theophylline		50	26	17	23	38	
10			33	23	19.5			
11			40	20	26	25	111	
12			45	15	20	23		
Average		36±1.7	42±3.5	21±2.3	20.6±2	23.6±0.5		
13	Sodium benzoate	36±1.7	40	17	20	23	23	29
14 to 27	None	36±1.7						

Dilution of Normal Rabbit Plasma with Human and Rabbit Fresh Plasmas Treated with $Al(OH)_3$ in Increasing Concentrations

The plasmas used to dilute the rabbit plasma were prepared by treatment with $Al(OH)_3$ to obtain concentrations of 0.01 *M*, 0.1 *M* and 0.2 *M* (8). The importance of albumins left in the human plasma was studied by making dilutions of the rabbit plasma with human albumins, obtained by precipitation of human plasma with $(NH_4)_2SO_4$, and then dialyzing until all the albumin fraction was ammonia-free (12). The results are recorded in Table III.

TABLE III

Dilution of Normal Rabbit Plasma with Human and Rabbit Fresh Plasmas, Treated with $Al(OH)_3$, on Increasing Concentrations and Its Influence on the Prothrombin Time

	Prothrombin time (sec.)
Normal rabbit plasma diluted 5% with rabbit alumina plasma 0.01 *M*	19
Normal rabbit plasma diluted 5% with rabbit alumina plasma 0.13 *M*	26
Normal rabbit plasma diluted 5% with human alumina plasma 0.01 *M*	20
Normal rabbit plasma diluted 5% with human alumina plasma 0.1 *M*	35
Normal rabbit plasma diluted 5% with human alumina plasma 0.2 *M*	42
Normal rabbit plasma diluted 5% with human albumin fraction from plasma	41
Normal rabbit plasma diluted 5% with distilled water or saline	32

Prothrombin-Free Caffeine Plasma and its Influence on the Prothrombin Time of Normal Rabbit Plasma

The plasma obtained from rabbits receiving caffeine was treated with 0.04 *M* $Al(OH)_3$, thus eliminating the prothrombin but not the thromboplastin cofactor. This plasma was then diluted to 10% with distilled water and 0.02 cc. were added to 0.1 cc. of normal rabbit plasma diluted to 5% with water. The prothrombin time of this 0.12 cc. was determined by the usual method. As a control, identical experiments were performed, using animals that had not received caffeine. The results are recorded in Table IV.

TABLE IV

Prothrombin Time of Normal Rabbit Plasma Diluted 5% plus 0.02 cc. of Normal or Caffeine Prothrombin-Free Rabbit Plasma Diluted 10% or 20%. Distilled Water Used to Dilute

	Prothrombin time (sec.)
Normal plasma diluted 5% + 0.02 cc. normal alumina plasma dil. 20%	28
Normal plasma diluted 5% + 0.02 cc. caffeine alumina plasma dil. 20%	21
Normal plasma diluted 5% + 0.02 cc. normal alumina plasma dil. 10%	35.5
Normal plasma diluted 5% + 0.02 cc. caffeine alumina plasma dil. 10%	19
Normal plasma diluted 5%	29

It is evident that prothrombin-free plasma from animals receiving caffeine can reduce the prothrombin time of 5% plasma from normal animals. The observation must be made with diluted normal plasma and diluted caffeine plasma, because the normal concentration of the thromboplastin cofactor is very high in rabbits. The dilution allows the investigation of any increase in this factor.

Prothrombin Time of Normal Rabbit Plasma Diluted to 5% with Human, Rabbit or Bovine Fibrinogen

Human and rabbit fibrinogen were diluted with saline to give the same concentration as the original plasma. Bovine fibrinogen was concentrated from 100 cc. of plasma to 16 cc. of saline. Then rabbit plasma was diluted with the fibrinogen solution

instead of saline, and the prothrombin time was determined. To study the real meaning of fibrinogen in the shortening of the prothrombin time, all the fibrinogen was eliminated from the fibrinogen solution. In the rabbit fibrinogen solution this was done with very small amounts of thrombin and then by heating to 60°C. for 10 min. to destroy the thrombin; in the bovine solution, the fibrinogen and the prothrombin were inactivated by heating to 58°C. The rabbit plasma was then diluted with these solutions. If the effect on the prothrombin time was maintained it would be the result of an impurity carried by the fibrinogen solution, but not the effect of fibrinogen itself. The results are recorded in Table V.

TABLE V

Prothrombin Time of Normal Rabbit Plasma Diluted with Human, Rabbit or Bovine Fibrinogen Solution

	Prothrombin time (sec.)
Normal plasma diluted 5% with rabbit fibrinogen solution	26
Normal plasma diluted 5% with rabbit fibrinogen solution after thrombin treatment and heating for 10 min. at 60°C.	23
Normal plasma diluted 5% with human fibrinogen solution	50
Normal plasma diluted 5% with bovine fibrinogen solution	15
Normal plasma diluted 5% with bovine fibrinogen solution heated to 58°C. and thus without fibrinogen and prothrombin	15
Normal plasma diluted 5% with distilled water or saline	35

Discussion

The shortening of the prothrombin time of rabbit plasmas under the influence of a single dose of caffeine, theobromine, theophylline or sodium benzoate is evident (Tables I and II). At the same time it is clear that these results can only be observed with plasma diluted to 10 and 5%. The reason why some investigators obtained different results is easy to explain on studying Table III. If dilution of normal rabbit plasma is made with human fresh plasma (instead of water or saline) treated with 0.01 *M* $Al(OH)_3$, the prothrombin time will be shorter than it should be, because this treatment does not eliminate the thromboplastin cofactor. This factor will produce an important error, because the normal values will be around 20 sec. instead of 35 or 36 sec. for 5% diluted plasma, and, therefore, no changes will be observed under the influence of drugs. But if the concentrations of $Al(OH)_3$ are increased, the prothrombin times obtained are completely different; with 0.1 *M* the prothrombin time is 35 sec. and with 0.2 *M* it is 42 sec.

In any case, it is indispensable to use an alumina solution with molarity enough to take out all the PCT. We suggest first trying the prothrombin time on plasma diluted with saline and then on plasma diluted with plasma treated with $Al(OH)_3$ of different concentrations. The correct molarity of alumina to be used must be that which can produce diluted plasmas giving a prothrombin time similar to that given by the saline dilution.

We think that the explanation for the increase of the prothrombin time on diluting with plasma treated with 0.2 *M* $Al(OH)_3$ beyond that for the saline-diluted is the adsorption of the thromboplastin cofactor (from the caffeine plasma) by the albumin of the diluting human plasma. The results obtained by diluting with the albumin fraction of human plasma instead of using the whole plasma treated with $Al(OH)_3$ permit this hypothesis (Table III).

From this part of the work it can be established that:

1. Dilution of human plasma with an insufficient amount of $Al(OH)_3$ fails to remove the thromboplastin cofactor, thus producing an increase of this factor in rabbit plasma and giving abnormally short prothrombin times.
2. Treatment with 0.1 *M* $Al(OH)_3$ produces values similar to those obtained on diluting with distilled water or saline, and the difference between the normal plasmas and the plasmas from rabbits receiving drugs are very clear.

But, as was demonstrated by us in a previous paper (8), the prothrombin time is the result of two factors, at least: the prothrombin itself and the plasma thromboplastin cofactor. Therefore, any change in the prothrombin time can be the result of an increase or decrease of any of these factors. The experiments recorded in Table IV prove that the shortening of the prothrombin time obtained under the influence of caffeine, theobromine, theophylline or sodium benzoate are not due to increased prothrombin activity (or levels) because the plasmas from rabbits receiving caffeine treated with $Al(OH)_3$ and thus without prothrombin (diluted to 10% with water), are able to reduce the prothrombin time of normal rabbit plasma (diluted to 5% with water) from 36 sec. to 19 sec. The same experiment, carried out with plasma from normal animals not receiving caffeine, gave 33 sec.

Table V demonstrates that fibrinogen cannot be the substance that produces the shortening of the prothrombin time. This is due rather to another fraction carried with it, because the total elimination of fibrinogen does not change the results. The increase of fibrinogen observed on

the animals receiving methylxanthines is a parallel phenomenon, but not the one responsible for the shortening of the prothrombin time.

From a study of this same table, it can be established that this factor, which we believe is the same one we have designated as the thromboplastin cofactor, is many times more concentrated in rabbit and bovine plasmas than in human plasma.

Summary

1. The influence of caffeine, theobromine, theophylline and sodium benzoate on the prothrombin time was studied.

2. The ability of these substances to shorten the prothrombin time is demonstrated.

3. If dilution of rabbit plasma is made with human fresh plasma treated with $Al(OH)_3$, this treatment must be done with 0.1 *M* solution to be sure that all the thromboplastin cofactor is eliminated. Special precautions must also be taken if fibrinogen solutions are used as a diluent.

4. The shortening of the prothrombin time is not due to increase of the prothrombin activity (or level) and the results obtained when dilution is made with fibrinogen solution are due to an impurity carried by the fibrinogen and not to the fibrinogen itself.

5. It is believed that these results are produced by the increase of the thromboplastin cofactor and that bovine and rabbit blood contain several times the amount present in human blood.

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Separation, Identification, and Estimation of Aromatic Food Preservatives and Sorbic Acid by Paper Chromatography and Ultraviolet Spectrophotometry

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Methods for the chromatographic separation and quantitative determination of some aromatic food preservatives have been published in *This Journal* (1, 2).

In this paper a method is described which permits the simultaneous separation, identification, and quantitative estimation of benzoic acid, the methyl, ethyl, propyl, and butyl esters of *p*-hydroxybenzoic acid, and sorbic acid. The separation is made by descending paper chromatography, and the positions of the compounds on the chromatogram are determined by taking contact prints in ultraviolet light. For further identification, and at the same time, quantitative estimation, the spots are eluted and the absorptions of the eluates are measured in a spectrophotometer.

METHOD

Apparatus and Reagents

(a) *Chromatographic tank.* — Rectangular tank, 19 × 29 × 60 cm. Place the solvent vessel 52 cm from the bottom of the tank.

(b) *Chromatographic solvent.*—Shake a mixture of *n*-butanol, ammonia, and water (5:2:3) in a separatory funnel. Place the bottom layer in a beaker in the bottom of the tank; use the top layer as the mobile solvent.

(c) *Filter paper.*—Whatman No. 1.

(d) *Ultraviolet lamp.*—Chromatolite Short Wave Lamp No. 2015 (Shandon), fitted with a OX7 filter to provide radiation at 253.7 mμ.

(e) *Photographic paper.*—Kodak Duostat 23, 29.7 × 42 cm sheets.

(f) *Standards.*—0.1 g of each acid or ester (or their sodium salts) in 100 ml of distilled water, ethanol/water (1:1), or 95% ethanol.

Procedure

Place 5-50 μl of the standard solution to be chromatographed along the base of a starting line drawn 7.5 cm from the top edge of the paper. Let the paper equilibrate in the tank for 1 hour; then add the solvent and let the chromatogram develop for 18-20 hours at 20°C. (The solvent front will travel a distance of 40-45 cm within this time.)

Remove the paper from the tank, mark the solvent front, and air-dry the paper in the hood. When dry, take a photographic print of the paper in ultraviolet light as described by Markham and Smith (3). Pin the paper chromatogram over a sheet of photographic paper on a table and expose it to the ultraviolet lamp for a few seconds (the time required to obtain good prints depends upon the distance between the paper and the lamp). On the developed print, the positions of the compounds chromatographed appear as white areas on a dark background, and may easily be traced on the chromatogram itself. Determine the R_f values in the usual way.

For further identification and quantitative determination cut the spot areas out of the paper together with congruent paper blanks positioned in the same areas on the paper as the spots themselves, and place in test tubes. Add measured amounts of 0.01*N* HCl (pH 2) or 0.01*N* NaOH (pH 12), and leave the tubes for at least 2 hours with occasional shaking. Read the absorbances of the eluates against the paper blank eluates in the region 200-300 mμ in a Beckman Model DU spectrophotometer or equivalent, and plot the spectra. (This step may be omitted if the R_f values are conclusive.)

Make a quantitative determination by reading absorbances (*A*) at the wavelengths of maximum absorption and calculate the concentrations of the compounds from their absorptivities, adjusting for dilutions. The molar absorptivity (*a*) is defined as: $a = A/bc$ where *b* is in cm and *c* in g/L.

Results

The R_f values of the compounds chromatographed are given in Table 1. The substances give very satisfactory photographic prints within reasonable concentrations, as shown in Fig. 1. The optimum quantities to be put on the paper were found to be as follows: benzoic acid, 25-250 μg; *p*-hydroxy-

benzoic acid esters, 5–100 μg; and sorbic acid, 2–25 μg (Fig. 1B).

It is well known that the aromatic food preservatives and sorbic acid all exhibit characteristic spectra in the ultraviolet region, owing to the presence of conjugated double bonds in their molecules. The shape and position of the spectra depend upon the pH of the solutions. The spectral properties may serve as a tool to verify the chromatographic identification of the compounds. Figure 2 shows spectra obtained with some food preservatives. As the curves show, there are profound spectral differences between the compounds, making the identification rather easy.

Table 1. R_f values of some food preservatives chromatographed at 22° C on Whatman No. 1 paper; solvent, n-butanol-ammonia-water (5:2:3), top layer

Compound	R_f
Benzoic acid	0.43
p-Hydroxybenzoic acid methyl ester	0.71
p-Hydroxybenzoic acid ethyl ester	0.80
p-Hydroxybenzoic acid propyl ester	0.84
p-Hydroxybenzoic acid butyl ester	0.87
Sorbic acid	0.51

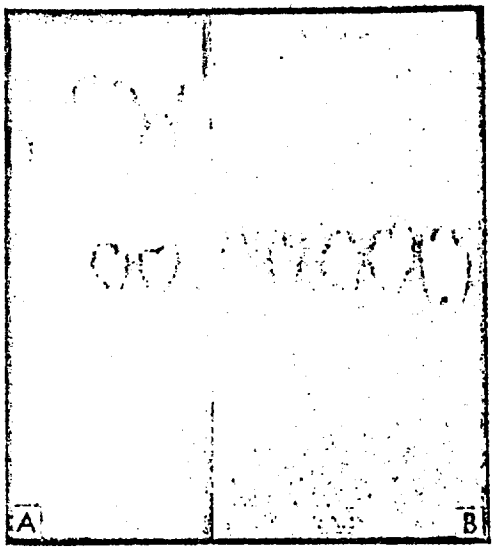

Fig. 1—(A) Ultraviolet contact print of food preservatives; left to right: p-hydroxybenzoic acid methyl ester, the butyl ester, sorbic acid, and a mixture of these three compounds. (B) Ultraviolet contact print of chromatogram with increasing amounts of sorbic acid; left to right: 1, 3, 5, 10, and 25 μg.

Table 2. Spectral characteristics of benzoic acid, its esters, and sorbic acid

Compound	pH 2						pH 12				
	λ max.	λ min.	$\frac{220}{260}$	$\frac{250}{260}$	$\frac{280}{260}$	ε max.	λ max.	λ min.	$\frac{220}{260}$	$\frac{250}{260}$	$\frac{280}{260}$
Sodium benzoate	230, 275	210, 261	11.2	1.84	1.25	11300	225, 269	210, 259	14.7	1.66	0.43
p-Hydroxybenzoic acid methyl ester (Na)	255	223	0.27	0.98	0.25	12800	219, 288	211, 242	0.72	0.43	2.06
p-Hydroxybenzoic acid ethyl ester (Na)	255	223	0.27	0.97	0.25	13650	222, 293	212, 242	0.76	0.41	2.42
p-Hydroxybenzoic acid propyl ester (Na)	257	223	0.21	0.85	0.27	13650	223, 294	213, 242	0.76	0.40	2.22
p-Hydroxybenzoic acid butyl ester	257	224	0.75	0.86	0.27	16800	222, 293	209, 244	2.61	0.24	4.85
Sorbic acid	255	212	0.92	0.98	0.23	26900	246	202	0.67	1.36	0.18

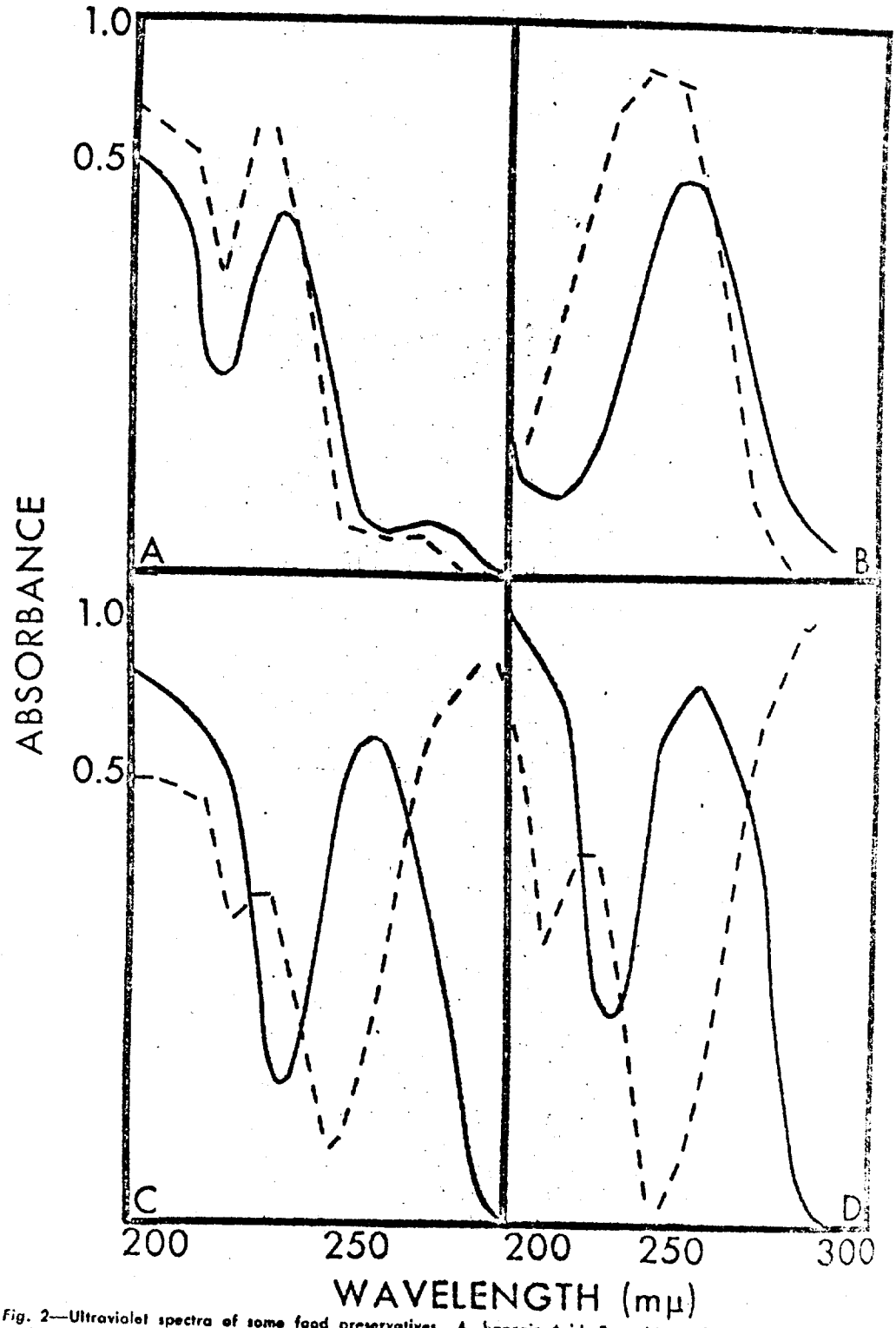

Fig. 2—Ultraviolet spectra of some food preservatives. A, benzoic Acid; B, sorbic acid, C, p-hydroxybenzoic acid methyl ester; D, p-hydroxybenzoic acid butyl ester. Spectra taken at pH 2 (———) and at pH 12 (·····) with a Beckman Model DB spectrophotometer.

Spectral characteristics are tentatively given in Table 2. Maxima and minima together with ratios A_{220} / A_{260} (220/260), A_{250} / A_{260} (250/260) and A_{280} / A_{260} (280/260) may give valuable information about the identity of the compounds. The absorptivities at maximum (a_{max}) are calculated for the compounds at pH 2.

Discussion

The chromatographic method described in this paper gives very satisfactory separation between some of the compounds. However, the separation of the four esters is rather poor, so that a mixture of the esters may not be separated sufficiently by this method. This difficulty may be partly overcome by using the smallest amounts of esters which are detectable by the method described here.

It is important not to exceed the optimal amounts of substances to be chromatographed. Too small amounts cannot be detected, while too large amounts overload the paper and cause severe tailing and false R_f values.

The detection of the spots by photographic contact printing is easy and rapid, but the exposure time is, of course, quite critical. Choosing the right exposure time is largely a matter of experience.

The spectra obtained are fairly specific, and are very good criteria of the identity of the compounds. However, they do not differentiate the esters; thus the only criterion of identity is the R_f value. The spectra of all the substances vary with the pH of the solution, and it is necessary to work at definite pH values. This is especially important in the quantitative determinations.

Although the method described here was originally developed to test the purity of commercial formulations of food preservatives, it should be readily applicable to the determination of UV-absorbing acids and esters in food. So far, the method has been found to work satisfactorily in the qualitative identification of the compounds in foods of different types. Its main advantage in this respect is that only small samples of food are required for analysis: 1 ml of liquid or 1 gram of solid food material is sufficient both for the qualitative and quantitative determination.

Further experiments, especially with regard to the quantitative determination, are being carried on in this laboratory, and detailed procedures for various types of food will be presented in a later publication.

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J. Am. Chem. Soc. 74: 5963-7, 1952.

[CONTRIBUTION NO. 1666 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The Enzyme-Inhibitor Dissociation Constants of α-Chymotrypsin and Several Series of Bifunctional Competitive Inhibitors[1]

BY H. T. HUANG AND CARL NIEMANN[2]

RECEIVED MARCH 28, 1952

The enzyme-inhibitor dissociation constants of α-chymotrypsin and several series of competitive inhibitors, *i.e.*, benzoic acid, phenylacetic acid, β-phenylpropionic acid, γ-phenylbutyric acid; the corresponding amides; β-indoleacetic acid, β-(β-indole)-propionic acid, γ-(β-indole)-butyric acid; β-(β-indole)-propionamide; and acetanilide, have been determined in aqueous media at 25° and *p*H 7.9 in the presence of a 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. A comparison of the results obtained in this study with those reported previously has shown that the affinity of α-chymotrypsin for certain negatively charged competitive inhibitors may be determined in part by the nature of the buffer present in the reaction system. The relation between the structure of certain of the above competitive inhibitors has also been considered and attention has been called to the possibility that each of the above inhibitors may combine with the active site of the enzyme in more than one way.

α-Amino acids or simple functional derivatives of these compounds that are specific substrates or competitive inhibitors of α-chymotrypsin may be described by the general formula $R_1CHR_2R_3$, where R_1, R_2 and R_3 are the three prominent structural features of these molecules, *i.e.*, the α-amino or acylamino group, the α-amino acid side chain and the carboxyl group or a functional derivative thereof.[3,4] It has been suggested[4] that the above specific substrates and competitive inhibitors may combine with the enzyme *via* an interaction involving the three groups R_1, R_2 and R_3, and a set of centers, ρ_1, ρ_2 and ρ_3 which are assumed to be a characteristic feature of the catalytically active site of the enzyme and which present a complementary aspect to molecules containing a particular set of R groups disposed in either one of the two possible configurations resulting from the asymmetry of the α-carbon atom present in these molecules. The extent to which any given compound of the general formula $R_1CHR_2R_3$ will be bonded to the active site of the enzyme will primarily depend upon the degree of complementariness originally obtaining between the molecule and the asymmetric catalytic surface, and secondarily, by the ability of both the combining molecule and the active site to alter their respective aspects to improve the closeness of fit during the course of the combining process.

The results of studies conducted in these laboratories on the behavior of specific substrates and competitive inhibitors of α-chymotrypsin have, so far, been consistent with the above view.[4-8] It may therefore be inferred that variations in the nature of disposition of the R groups will be reflected by changes in the corresponding enzyme-inhibitor or enzyme-substrate dissociation constants even in the case of bifunctional compounds where one of the R groups is replaced by a sterically unimportant hydrogen atom. Thus with bifunctional competitive inhibitors of the general formula $R_1(CH_2)_nR_2$, $R_1(CH_2)_nR_3$ and $R_2(CH_2)_nR_3$ it should be possible not only to determine the optimum value of *n* for the best fit to the complementary surface at the active site of the enzyme but also to make a rational estimate of the relative importance of various R groups in the over-all binding process.

In this study we have chosen for examination a group of bifunctional competitive inhibitors R_2-$(CH_2)_nR_3$ where R_2 = benzyl or β-indolylmethyl and R_3 = carboxylate or carbamido. When this work was initiated, one member of this group of compounds, *i.e.*, β-phenylpropionic acid, had already been studied[9] although the data were inadequate to establish the nature of the inhibition. The present experiments were all performed at 25° and *p*H 7.9 in aqueous solutions 0.02 *M* with respect to the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. In all cases the *p*H of the medium remained within the desired range, *i.e.*, 7.9 ± 0.2 even when the inhibitors containing a carboxylate group were introduced in the form of their sodium salts. Except where noted nicotinyl-L-tryptophanamide was used as the specific substrate with an enzyme concentration corresponding to 0.208 mg. of protein nitrogen per ml. Since in every instance the hydrolytic reaction was limited to within 30% hydrolysis all of the systems investigated can be formulated in terms of equations (1) and (2) where $K_S = (k_2 + k_3)/k_1$ and $K_I = k_5/k_4$.[10] The

$$E_t + S_t \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E_t + P_{1t} + P_{2t} \qquad (1)$$

$$E_t + I_t \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EI \qquad (2)$$

(1) Supported in part by a grant from Eli Lilly and Company.

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(6) H. J. Shine and C. Niemann, THIS JOURNAL, 74, 97 (1952).

(7) H. T. Huang and C. Niemann, *ibid.*, 74, 101 (1952).

(8) H. T. Huang, R. J. Foster and C. Niemann, *ibid.*, 74, 105 (1952).

(9) S. Kaufman and H. Neurath, *J. Biol. Chem.*, 181, 623 (1949).

(10) For definition of symbols *cf.* H. T. Huang and C. Niemann, THIS JOURNAL, 73, 1511 (1951).

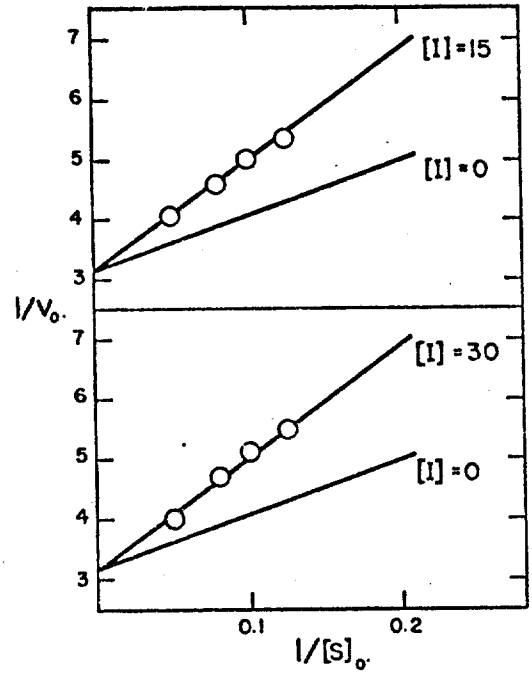

Fig. 1.—Plot of equation (3) for the determination of the K_I values of the first group of inhibitors: v_0 in units of 10^{-3} M per min., $[S]_0$ in units of 10^{-3} M nicotinyl-L-tryptophanamide, [E] equivalent to 0.208 mg. protein-nitrogen per ml.: upper curve, $[I] = 15 \times 10^{-3}$ M β-(β-indole)-propionate; lower curve, $[I] = 30 \times 10^{-3}$ M β-indoleacetate.

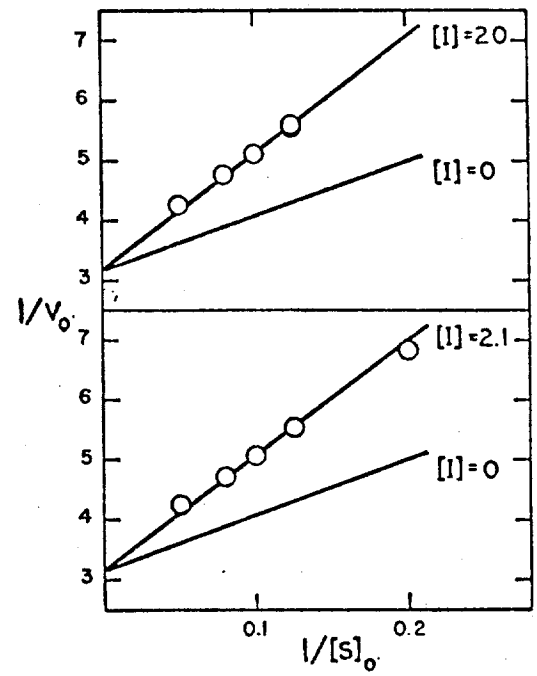

Fig. 2.—Plot of equation (3) for the determination of the K_I values of the second group of inhibitors: v_0 in units of 10^{-3} M per min., $[S]_0$ in units of 10^{-3} M nicotinyl-L-tryptophanamide, [E] equivalent to 0.208 mg. protein-nitrogen per ml.: upper curve, $[I] = 20 \times 10^{-3}$ M γ-(β-indole)-butyrate; lower curve, $[I] = 2.1 \times 10^{-3}$ M β-(β-indole)-propionamide.

inhibitor concentration used for each individual inhibitor was selected so as to provide a degree of inhibition convenient for the evaluation of K_I from a plot of $1/v_0$ *versus* $1/[S]_0$ based upon equation (3).[7,11]

$$\frac{1}{v} = \frac{K_S}{V}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V} \qquad (3)$$

The data obtained in the present investigation are summarized in Figs. 1–5 and in Table I. The plots given in Figs. 1–5 leave no doubt that all of the inhibitors studied are competitive inhibitors and since K_I is a true equilibrium constant the standard free energy changes for the formation of the various enzyme inhibitor complexes have been calculated. These latter values are given in Table I along with the corresponding enzyme-inhibitor dissociation constants.

TABLE I

ENZYME-INHIBITOR DISSOCIATION CONSTANTS

Inhibitor	H and N K_I[b]	H and N $-\Delta F°$[c]	N and G[a] K_I[d]	N and G[a] $-\Delta F°$[c]
β-Indoleacetate	25	2180		
β-(β-Indole)-propionate	13	2570	2.5	3570
γ-(β-Indole)-butyrate	17	2410	3.6	3350
Benzoate	200	950	42	1880
Phenylacetate	120	1250	42	1880
β-Phenylpropionate	28	2120	5.5	3080
γ-Phenylbutyrate	72	1560	14	2530
β-(β-Indole)-propionamide	1.7	3780		
Benzamide	6.6	2970		
Phenylacetamide	10.2	2720		
β-Phenylpropionamide	6.7	2960		
γ-Phenylbutyramide	7.2	2920		
Acetanilide	10.4	2700		

[a] *Cf.* ref. 12. [b] In units of 10^{-3} M at 25° and pH 7.9 in systems 0.02 M in respect to the amine component of a tris-(hydroxymethyl)-aminomethane-hydrochloric acid buffer. [c] In cal. per mole to the nearest 10 cal. [d] In units of 10^{-3} M at 25° and pH 7.8 in systems 0.1 M in a phosphate buffer.

Because of the very large difference between the value of K_I which we obtained for β-phenylpropionic acid, *i.e.*, 28×10^{-3} M, and the value reported by Kaufman and Neurath,[9] *i.e.*, 4.5×10^{-3} M, it was thought desirable to check our value with another specific substrate. Two experiments with $[S]_0 = 20 \times 10^{-3}$ M acetyl-L-tyrosinamide and $[I] = 30 \times 10^{-3}$ M gave a value for K_I of β-phenylpropionic acid, under the conditions previously specified, of approximately 30×10^{-3} M. That the difference between our value and that of Kaufman and Neurath[9] must reflect a fundamental difference between the systems we had studied and those investigated by Kaufman and Neurath[9] became evident when, during the final stages of the present investigation, a paper by Neurath and Gladner[12] appeared, in which K_I values were reported for six of the inhibitors listed in Table I, including a revised value for β phenylpropionic acid, *i.e.*, 5.5×10^{-3} M. The values of Neurath and Gladner were determined from $1/v_0$ *versus* $1/[S]_0$ plots using data obtained from aqueous systems at 25° and pH 7.8 in which acetyl-L-tyrosinamide was the specific substrate and 0.1 M phosphate the buffer. It is apparent that for every one of the four inhibitors, β-phenylpropionic acid, γ-

(11) H. Lineweaver and D. Burk, THIS JOURNAL, 56, 658 (1934).

(12) H. Neurath and J. A. Gladner, *J. Biol. Chem.*, 188, 407 (1951).

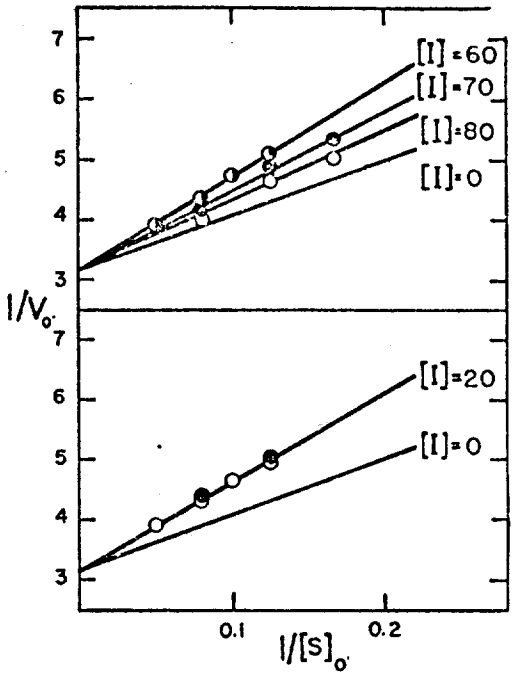

Fig. 3.—Plot of equation (3) for the determination of the K_I values of the third group of inhibitors: v_0 in units of 10^{-3} M per min., $[S]_0$ in units of 10^{-3} M nicotinyl-L-tryptophanamide, [E] equivalent to 0.208 mg. protein-nitrogen per ml.: upper curve, ◑, [I] = 60 × 10^{-3} M γ-phenylbutyrate; ●, [I] = 70 × 10^{-3} M phenylacetate; O, [I] = 80 × 10^{-3} M benzoate; lower curve, [I] = 20 × 10^{-3} M β-phenylpropionate.

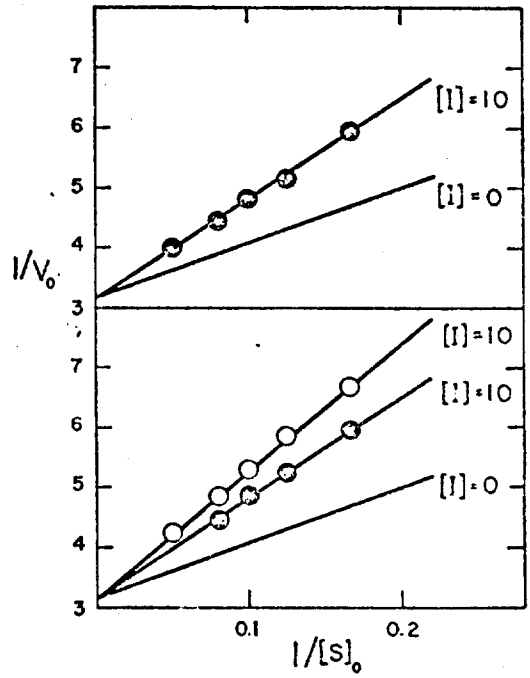

Fig. 4.—Plot of equation (3) for the determination of the K_I values of the fourth group of inhibitors: v_0 in units of 10^{-3} M per min., $[S]_0$ in units of 10^{-3} M nicotinyl-L-tryptophanamide, [E] equivalent to 0.208 mg. protein-nitrogen per ml.: upper curve, [I] = 10 × 10^{-3} M acetanilide; lower curve, O, [I] = 10 × 10^{-3} M benzamide; ●, [I] = 10 × 10^{-3} M phenylacetamide.

phenylbutyric acid, β-(β-indole)-propionic acid and γ-(β-indole)-butyric acid, where it has been possible for us to evaluate K_I at near optimum inhibitor concentration,[7] our values, *i.e.*, 28, 72, 13 and 17 × 10^{-3} M are very nearly five times those given by Neurath and Gladner,[12] *i.e.*, 5.5, 14, 2.5 and 3.6 × 10^{-3} M.[13] The difference between our values for competitive inhibitors of the type $R_2(CH_2)_nR_3$, where R_2 = benzyl or β-indolymethyl and R_3 = carboxylate and those of Neurath and Gladner[12] corresponds to a decrease in $-\Delta F^\circ$, in passing from our reaction system to that of Neurath and Gladner,[12] of approximately 1000 cal. per mole, which is a large fraction of the $-\Delta F^\circ$ values encountered for the formation of the enzyme-inhibitor complexes of all competitive inhibitors that have so far been studied in detail.[4,6-10,12,14-18]

The relatively good agreement between the K_S value of acetyl-L-tyrosinamide determined in systems 0.02 M in the amine component of a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer and that obtained in systems 0.1 M in a phosphate buffer[14] gives no indication that this value is

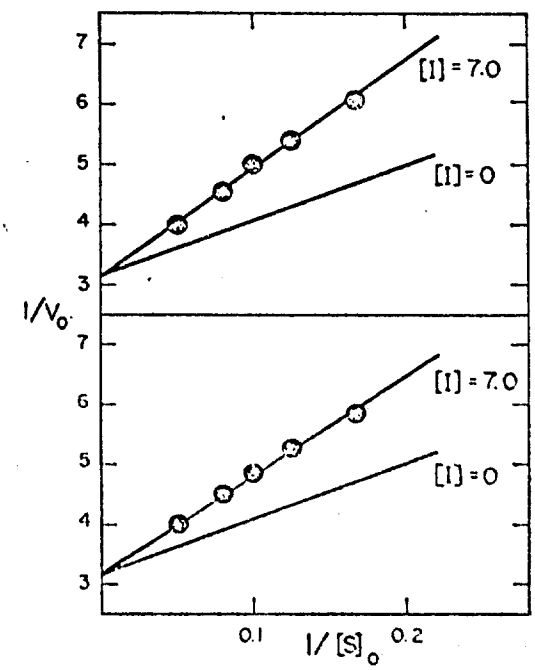

Fig. 5.—Plot of equation (3) for the determination of the K_I values of a fifth group of inhibitors: v_0 in units of 10^{-3} M per min., $[S]_0$ in units of 10^{-3} M nicotinyl-L-tryptophanamide, [E] equivalent to 0.208 mg. protein-nitrogen per ml.: upper curve, [I] = 7 × 10^{-3} M β phenylpropionamide; lower curve, [I] = 7 × 10^{-3} M γ-phenylbutyramide.

markedly sensitive to the above changes in ionic strength or ion species. A further indication of the

(13) It is felt that our values for benzoic acid and phenylacetic acid are not sufficiently accurate for quantitative comparisons, since the measured increases in the slopes of the $1/v_0$ *versus* $1/[S]_0$ plots are very small in these cases, *cf.* Fig. 3.

(14) D. W. Thomas, R. V. MacAllister and C. Niemann, THIS JOURNAL, 73, 1548 (1951).

(15) H. T. Huang and C. Niemann, *ibid.*, 73, 1555 (1951).

(16) H. T. Huang and C. Niemann, *ibid.*, 73, 3228 (1951).

(17) H. T. Huang, R. V. MacAllister, D. W. Thomas and C. Niemann, *ibid.*, 73, 3231 (1951).

(18) H. T. Huang and C. Niemann, *ibid.*, 73, 4039 (1951).

lack of sensitivity of the K_S values of certain specific substrates to changes in ionic strength or ion species is to be found in the excellent agreement between the K_S value of glycyl-L-tyrosinamide determined at *p*H 7.8 in a tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer of unspecified concentration, *i.e.*, 120×10^{-3} *M*,[19] and the value obtained at *p*H 7.8 in a 0.1 *M* phosphate buffer, *i.e.*, 122×10^{-3} *M*.[20] However, it is conceivable that a change in ionic strength could have a far more pronounced effect upon the K_I values of carboxylic acids. An increase in ionic strength could cause a decrease in the activity coefficient of the inhibitor anion and hence lead to an increase in the K_I value. On the other hand if, as it seems likely, a negative charge is situated in the vicinity of the catalytically active site,[3] the approach of a negatively charged inhibitor ion would be enhanced by this increase in ionic strength, thus causing a decrease in the K_I value. While the balance of these two opposing effects may be in favor of combination, it is difficult to see from these considerations how an increase in ionic strength, within the limits involved, can be solely responsible for the difference noted in the two buffer systems. Therefore, it appears that the difference in the two sets of K_I values, for the four bifunctional anionic competitive inhibitors being compared, may be due to a specific ion effect that is operative in one or both of the buffer systems and which profoundly influences the nature of the reaction occurring between the enzyme and certain negatively charged competitive inhibitors of the hydrolytic reaction.

Despite the fivefold discrepancy in the two sets of K_I values for the carboxylic acids listed in Table I, it is clear that our results are in excellent agreement with those of Neurath and Gladner[12] with respect to the importance of the degree of separation of the R_2 and R_3 groups in determining the affinity of the enzyme for inhibitors of the general formula $R_2(CH_2)_nR_3$. It is interesting to note that the K_I value of β-(β-indole)-propionamide, 1.7×10^{-3} *M*, is actually less than both the K_S value of acetyl-L-tryptophanamide, 5.3×10^{-3} *M*, and the K_I value of acetyl-D-tryptophanamide, 2.7×10^{-3} *M*.[10] Similarly, the K_I value of β-phenylpropionamide, 6.7×10^{-3} *M*, is less than both the K_S value of acetyl-L-phenylalaninamide, 34×10^{-3} *M*,[8] and the K_I value of acetyl-D-phenylalaninamide, 14×10^{-3} *M*.[8] These results are in accord with the idea, expressed previously,[3,7] that the strain present in an intermediate complex derived from a trifunctional interaction, *i.e.*, R_1–ρ_1, R_2–ρ_2 and R_3–ρ_3, is greater than that in one involving a bifunctional interaction, *e.g.*, R_2–ρ_2 and R_3–ρ_3. They also indicate that the acetamido and carbamido functions are inherently groups of low affinity for the enzymatic site, in contrast to the β-indolylmethyl and benzyl groups, which are apparently primarily responsible for the favorable binding free energy in the interaction between the enzyme and the above inhibitor.

The value of K_I for β-(β-indole)-propionate, 13×10^{-3} *M*, lies between the K_I value of acetyl-L-tryptophanate, 17.5×10^{-3} *M*,[10] and that of acetyl-D-tryptophanate, 4.8×10^{-3} *M*,[4] while the K_I value of β-(β-indole)-propionamide, 1.7×10^{-3} *M*, is less than both the K_S value of acetyl-L-tryptophanamide, 5.3×10^{-3} *M*,[10] and the K_I value of acetyl-D-tryptophanamide, 2.7×10^{-3} *M*.[10] These results suggest that when R_3 = carboxylate this group has very little affinity for the corresponding ρ_3 center in systems containing a 0.02 *M* tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer. Thus, when R_3 = carboxylate, in spite of the expectation that some strain will be created by the interaction of the three R groups of acetyl-D-tryptophanate with the corresponding ρ centers of the catalytically active site, the attractive forces, principally due to the interaction of R_1 and ρ_1 and R_2 and ρ_2, result in a more favorable $-\Delta F^\circ$ than in the case of β-(β-indole)-propionate, where only one interaction, *i.e.*, between R_2 and ρ_2, can effectively contribute to the binding energy. However, when R_3 is a carbamido group the interaction of R_3 and ρ_3 clearly results in an attraction and this added increment contributes more effectively to the binding energy of the bifunctional β-(β-indole)-propionamide, *i.e.*, 3780 cal., than to that of the trifunctional acetyl-D-tryptophanamide, *i.e.*, 3500 cal.,[10] and actually results in a greater $-\Delta F^\circ$ for the former compound.

With the 0.02 *M*-tris-(hydroxymethyl)-aminomethane–hydrochloric acid buffer the difference in $-\Delta F^\circ$ between the two pairs β-(β-indole)-propionamide and β-(β-indole)-propionate and β-phenylpropionamide and β-phenylpropionate is 1600 and 840 cal., respectively. However, if the $-\Delta F^\circ$ values of Neurath and Gladner,[12] which were determined in systems containing a 0.1 *M* phosphate buffer, are used for the above carboxylate ions the difference, in $-\Delta F^\circ$, is 210 and -120 cal., respectively. Thus it appears that the 0.1 *M* phosphate buffer is capable of increasing the affinity of the enzyme for these carboxylate ions to the point where the difference observed in the first buffer system, is, for all practical purposes, abolished. In this connection it is of interest to note that Neurath and Gladner[12] found that in the 0.1 *M* phosphate buffer the K_I values of β-phenylpropionate and β-phenoxyethanol were 5.5 and 5.8×10^{-3} *M*, respectively.

In the series $R_2(CH_2)_nR_3$ where R_2 = benzyl and R_3 = carbamido and n = 0, 1 and 2, it is seen that the most effective inhibitor is obtained when n = 1, and that the affinity is decreased when n is changed to 0 or 2. The decrease in affinity is, however, much less pronounced than that observed for the same changes where R_2 = benzyl and R_3 = carboxylate. Furthermore, benzamide, which can be regarded either as a member of the above series with $n = -1$, *i.e.*, as a R_2 group, or as a benzamido derivative, *i.e.*, as a R_1 group, has about the same K_I value as that of β-phenylpropionamide. Apart from further emphasizing the relatively low affinity of the carbamido group for ρ_3, these considerations suggest that our interpretation of the mode of combination of α-chymotrypsin with competitive inhibitors of this enzyme may be grossly oversimplified

(19) D. C. Doherty and F. Vaslow, THIS JOURNAL, 74, 931 (1952).

(20) S. Kaufman, H. Neurath and G. W. Schwert, *J. Biol. Chem.*, 177, 793 (1949).

particularly when applied to bi- and monofunctional inhibitors. If it is assumed that the benzamido group in benzamide can interact with the center ρ_2, it is also necessary to assume that it can interact with the center ρ_1.[4,7] While it is conceivable that benzamide may interact with one center more readily than with the other, both of these intermediate complexes would block combination of the catalytically active site of the enzyme with trifunctional specific substrates such as nicotinyl-L-tryptophanamide and acetyl-L-tyrosinamide. Therefore the experimental value of K_I for benzamide could be a function of two separate dissociation constants, one for each of the above two intermediate complexes. The situation may be even more complicated since the existence of a third complex based upon simultaneous interaction of two molecules of benzamide with the centers ρ_1 and ρ_2 cannot be entirely excluded. Thus the relatively low value of K_I for benzamide may reflect an enhanced probability of combination arising from a multiplicity of modes of combination rather than a high affinity in any particular mode. At present it is not possible to evaluate quantitatively the relative contributions of each of the three types of intermediate complexes that may be formed.

While it may be fortuitous that the K_I value of acetanilide, 10.4×10^{-3} M, is practically identical with that of phenylacetamide, 10.2×10^{-3} M, it does appear that with these two isomeric competitive inhibitors a transposition of the $-CH_2-$ and $-NH-$ groups present in their side chains is without demonstrable effect upon the over-all binding process. This indicates that with these two compounds the principal factor in the combination process is the size and shape of the molecule, and hence a van der Waals interaction.

The data obtained during the course of this investigation have pointed out the existence of two fundamental aspects of certain enzyme-inhibitor and enzyme-substrate interactions involving α-chymotrypsin, *viz.*, (a) the fact that the affinity of the enzyme for certain anionic competitive inhibitors may be profoundly influenced by the nature of the buffer present in the reaction system, and (b) that several different modes of combination may be involved in the interaction of this enzyme with a given specific substrate or competitive inhibitor. Since both of these issues are important for a correct understanding of available kinetic data it is hoped that studies now in progress will expand our knowledge along these lines.

Experimental[21,22]

Competitive Inhibitors.—β-Indoleacetic acid, m.p. 167–168°; β-(β-indole)-propionic acid, m.p. 133–134°; γ-(β-indole)-butyric acid, m.p. 123–124°; benzoic acid, m.p. 122–123°; phenylacetic acid, m.p. 76–77°; β-phenylpropionic acid, m.p. 48–49°; γ-phenylbutyric acid, m.p. 50–51°; acetanilide, m.p. 113–114°; and benzamide, m.p. 129–130°, were Eastman Kodak Co. products which were recrystallized at least twice from appropriate solvents. Phenylacetamide, m.p. 157–158°; β-phenylpropionamide, m.p. 105–106°, and γ-phenylbutyramide, m.p. 84–85°, were prepared from the corresponding acids by ammonolysis of the respective acid chlorides. Ammonolysis of 3 g. of crude methyl β-(β-indole)-propionate, prepared by the reaction of methanolic hydrogen chloride with the corresponding acid, gave 1.5 g. of β-(β-indole)-propionamide, fine stunted needles, m.p. 204–205°.

Anal. Calcd. for $C_{11}H_{12}ON_2$ (188): C, 70.3; H, 6.4; N, 14.9. Found: C, 70.3; H, 6.2; N, 14.9.

Enzyme Experiments.—The methods used were identical with those described previously. The preparation of the principal specific substrate, *i.e.*, nicotinyl-L-tryptophanamide for which $K_S = 2.7 \times 10^{-3}$ M,[10] has also been described.[23] The enzyme preparation was an Armour product, lot no. 90402, and it will be noted that the specific enzyme concentrations E'_S and E'_I for all experiments were such as to satisfy zone A conditions.[10,24,25]

(21) Microanalyses by Dr. A. Elek.

(22) All melting points are corrected.

(23) B. M. Iselin, H. T. Huang, R. V. MacAllister and C. Niemann, THIS JOURNAL, **72**, 1729 (1950).

(24) O. H. Straus and A. Goldstein, *J. Gen. Physiol.*, **26**, 559 (1943).

(25) A. Goldstein, *ibid.*, **27**, 529 (1944).

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Voprosy Pitaniya 24(3): 61-68, 1965.

EXPERIMENTAL DATA ON THE HYGIENIC CHARACTERISTICS OF THE COMBINED ACTION OF CERTAIN CHEMICAL FOOD PRESERVATIVES

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The goals of our scientific investigations were as follows: the deeper study and comparison of the toxicity of sorbic acid in Czechoslovak and Soviet industry (Laboratory and production-line), also of polymer admixtures to the latter product; to confirm foreign literature on the relative absense of nisin toxicity in British production (currently in the Soviet Union semiindustrial testing of nisin is ongoing and the possibility of obtaining the parent substance is under study);deeper research into the degree of expression of toxic properties in domestic industrial preservatives in current use--sodium bisulfite and benzoic acid; to study the combined action on the animal organism of sorbic acid+ nisin and sodium bisulfite + benzoic acid.

In the experiment, pure preservative preparations were used. the preparation doses were computed based on their proper concentrations in food products at a safety factor(SF) of 1 and 2. Thus, 40 and 80 mg of sorbic and benzoic acids were given and 80 and 160 mg of sodium bisulfite, nisin-- 2 and 4 mg/kg animal body weight. Additionally, 0.4,40, 400 mg/kg doses of nisin were studied and 0.4 and 800 mg/kg doses of the polymer. Acute tests were not done and subacute experiments were done to a limited degree. The basic emphasis was on chronic experiments.

1900 white mice and 400 white rats of both sexes and varying weights(in agreement eith literature data of preservative studies by other authors, mice were not used). In chronic experiments, the compounds were taken by the experimental animals in a group dosage method. For this we prepared innoculating mixtures consisting of the test substances and products and included them in the animals' food. The control animals ate the same mixtures but without preservatives. The mixtures were given separately from the general diet.

Statistical treatment of the data obtained was by the variational statistical method. We determined the average arithmetical, quadrant deviation from the average,average parameter error, differential error indicators and reliability criteria,coefficient of correlation and the dependence of the expedient use of corresponding statistical methods.

Three test series were done. The duration of the chronic experiments was 8--15 months and of the subacute,3 months.

In studying the animals, we employed more than 30 criteria: observation of general condition, production of offspring, viability under general experimental conditions and during starvation, utilization of water and food, weight gain, tests of cooling of the body, physical stress, tests with CCl_4 and K_2HPO_4,blood formula determinations, complement titer,phagocytic activity of leucocytes and blood oxidase activity, ESR according to Erman, blood alkalinity and ke-

tone bodies in the blood, protein in urine, histopathological investigations and investigation of the possibility of carcinogenic action of the indicated substances.

The animals were divided into the following groups: K--control; A-- those receiving a mixture of sorbic acid and nisin; B-- those receiving a mixture of sodium bisulfite and benzoic acid; C--nisin; D--sorbic acid; E-- sodium bisulfite, and F--benzoic acid.

The experiments showed that the highest absolute and relative body weight increase and also the utilization of water and food during simultaneous decrease in viability under ordinary experimental conditions and also during fasting occurred in mouse and rat males who had received nisin (Group C). According to most indicators, sorbic acid was an antagonist to nisin. Thus, in group A, body weight, viability and use of food and water was within the same limits ad for the control group, and in Group Dthe viability of mice was 8% higher than for the control group. In animals, especially with mice, who received sodium bisulfite and benzoic acid(separately and mixed) the indicating signs were lower than for controls. Benzoic acid exertad a negative effect on body weight and viability of mice (for rats, this did not occur). Results are shown in tables 1--4.

From the data shown in table 4 it is evident that the lowest viability of male rats during starvation was found in group C. In comparison with group D (there were no males in the control group) this difference was statistically demonstrable and should be clearly the effect of the preservative, such that χ^2 for the initial weight and life expectancy of fasting rats was more than 99%.

Experiments involving physical stress (swimming) involved the use of weights tied to the tails of the mice and weighing either 5 or 50% of body weight. The results are presented in table 5.

From table 5 it is evident that the most tolerant to physical stress was found among female mice in groups A and D (at 5% body weight added). In comparison with group K, B and E, this difference in statistical reliability was established at $P<0.001$, <0.05, <0.05. At this level there was no demonstrable relationship between mouse body weight and endurance of physical load, that permits the removal of a difference in duration of remaining at the water surface at the expense of the action on the substance of the indicated preservatives.

In 8 out of 100 mice of group B, spontaneous tumors were observed and in the third generation of the 8 mice at the same time they were not observed in the other groups. In connection with the obtained data we conducted a study of possible carcinogenic effects of these preservatives and also on the influence of excess body weight on the speed of development of innoculated intestinal Erlich ascites tumor cells. The results of these studies will be published separately. According to the data obtained, benzoic acid and sodium bisulfite, both by themselves and in mixtures showed carcinogenic effects under known conditions.

To determine the general detoxification ability of the liver we applied the stress of CCl_4: for mice 0.1 ml and for rats 0.6 ml. Based on a single dose per os, sodium bisulfite and benzoic acid

and nisin lowered and sorbic acid and polymer increased the detoxification capacity of the liver . Statistical data are reliable.

As a general indicator of the total immunological state of the experimental animals, we used determinations of the complement titer and pagocytic activity of leucocytes; forcharacteristic levels of oxidative processes, and also of the functional condition of the liver--the determination of blood oxidase activity.

We determined complement titer by the classical method by tenfold reagent dilution and twentyfold blood serum dilution. The phagocytic activity of leucocytes was also determined by classical means using a 2-billion innoculum of Staphylococcus strain 207. Blood oxidative activity was determined using paraphenylendiamine according to Ravin [1]. For these tests we used 10--20 rats from each group.

However in connection with the absense of statistically reliable shifts in the study of the effect of preservatives on the animals under stress at the end of the chronic experiment, all animals were additionally divided into subgroups and subjected to cold (in the winter) and centrifugal stress in a rotating drum.There appeared such functional disruptions as altered kidney function under K_2HPO_4. The results of these studies are shown in table 6.

The data in table 6 shows that the highest indicators were in groups A and D for the general experiments. In comparison with group B this difference was statistically reliable, especially for complement titer ($P < 0.001$). In group E there was very low leucocyte phagocytic activity in the blood. Group F results were within control limits.

Cold and centifugal stress had more negative effect on group B (in comparison with group A),because all indicators decreased. The experiment with the effects of cold conducted after 8 months gave identical results: in group A oxidase activity was almost unchanged (optical density decreased by 0.030), and for B it decreased 0.282. In subacute experiments (FB-20) oxidase activity of the blood was higher for group A than for group B (optical density read at beginning and end of experiment: for group A--0.196, 0.442; in group B--0.161 and 0.623). These data show that these preservative mixtures affect oxidative processes in the animals. If sorbic acid + nisin in all cases increases blood oxidase activity, under biologically pleasant conditions, then sodium bisulfite (separately and mixed with benzoic acid) in small doses lowers and in large doses. sharply raises it, that in both conditions,biologically unbeneficial properties of a given preservative appear.

Changes noted in the blood oxidase activity in chronic experiments suggest the presence of possible shifts in the levels of acetone bodies in the blood. Acetone bodies determined in rats by Natelson's method [2] in group K was 2.5 mg%, in group A--3.4mg%, and in group B--1.97mg%. However, two weeks later , these rats were subjected to subacute tests (FB-20)and observation of the blood acetone content in these animals led to the following results: Group K--1.53 mg%, group A--2.15mg%, and group B--2.88 mg%. This shows that increasing the sorbic acid and nisin dose does not cause increased levels of acetone bodies in the blood while larger doses of sodium bisulfite and benzoic acid raise this level.

TABLE I

WEIGHT INCREASE OF MICE AFTER EIGHT MONTHS OF TESTS (SF-1)

Group and sex of mice		basic weight 8--15 g			basic weight 16--25 g		
		* in g	** in %		in g	in %	
K	males	26.0	185.0	94.40	10.74	53.50	42.47
	females	11.16	97.6	68.13	15.20	90.03	68.44
A	males	28.0	257.0	108.60	18.31	91.03	66.37
	females	19.3	160.0	93.30	13.90	72.02	55.29
B	males	23.67	179.0	105.12	15.55	77.60	67.00
	females	11.01	108.0	73.67	13.39	72.10	56.14

* absolute weight gain
** relative weight gain in %

TABLE II

WEIGHT GAIN IN RATS AFTER SEVENTEEN MONTHS OF TESTS (SF-1)

group and sex of rats		*	absolute change in g	absolute change in %	**
K	males	111,88	227,65	203,49	111,0
	females	101,2	170,8	168,77	98,93
A	males	101,79	258,21	253,67	126,39
	females	97,54	198,0	202,99	110,85
B	males	116,16	191,48	164,84	97,41
	females	98,40	167,06	151,80	99,42

* initial wt. in g.
**relative change (in%)

TABLE III

BODY WEIGHT, FOOD UPTAKE (PELLETS), WATER UPTAKE, VIABILITY DURING THE EXPERIMENTAL PERIOD AND DURING FASTING FOR MICE (SP-2)

mouse group and sex		absolute gain in g	absolute gain in %	relative gain in %	uptake/day Food g.	uptake/day ml. water	viability *	viability **
A	males	16.57	199.39	109.7	2.33	3.67	60	3.86
	females	12.13	166.83	93.26	2.81	4.14	72	5.22
A	males	12.19	128.72	82.74	2.34	3.32	48	10.0
	females	10.68	114.59	78.35	2.48	4.42	74	5.71
B	males	16.94	174.3	100.9	3.24	3.54	30	3.67
	females	12.24	140.69	87.85	2.32	2.63	38	4.67
C	males	13.35	145.44	89.78	2.17	3.87	92	4.28
	females	13.47	141.47	89.36	2.13	3.31	52	4.72
D	males	19.20	226.85	118.45	3.36	5.92	12	3.52
	females	12.47	143.33	87.99	4.31	4.34	32	4.57
E	males	13.70	143.61	88.80	2.43	3.38	70	4.71
	females	11.21	124.14	80.71	1.98	3.13	68	5.64
F	males	11.28	124.52	80.87	2.57	3.85	68	4.4
	females	10.95	118.23	78.05	2.65	3.52	68	4.4

*during experimental period %
**during fasting(in days)

TABLE IV

RESULTS OF FASTING EXPERIMENTS WITH RATS (SF-2)

Group and sex (rats)		*	**	***	****
K	females	179,5	36,21	9,75	>0,05
D	males	225,2	43,7	12,2	
	females	170,5	38,65	9,5	
F	males	221,2	43,4	11,8	>0,1
	females	162,4	39,2	8,8	>0,5
F	males	193,6	41,0	12,2	
	females	176,8	36,2	12,0	
C	males	185,5	39,1	9,25	<0,05
	females	173,0	37,6	9,5	

*initial body weight (in g)
**weight loss in %
***viability (in days)
****reliability (P)

TABLE V

ENDURANCE OF PHYSICAL LOAD IN MICE (SF-1)

group and sex	mice	*	**
		5%	50%
K	male	4,66	5,0
	female	5,17	5,5
A	male	5,78	27,25
	female	26,1	6,52
B	male	11,9	16,33
	female	13,71	13,55
D	male	11,67	—
	female	24,17	—
E	male	2,6	7,0
	female	7,54	5,42
F	male	11,71	—
	female	16,33	—

*swimming time in min

**added weight per total body weight

TABLE VI

COMPLEMENT TITER, PHAGOCYTIC ACTIVITY OF LEUCOCYTES AND OXIDASE ACTIVITY OF THE BLOOD DURING 17 MONTHS FROM THE BEGINNING OF THE EXPERIMENT IN RATS, MAINTAINED UNDER THE GENERAL CONDITIONS OF A VIVARIUM AND SUBJECTED TO THE ACTION OF STRESS FACTORS

RAT group	experimental conditions	complement titer		phagocytosis		oxidase activity optical density of samples
		in centimilliliters	than 2 centimilliliters	%	index	
К	Контроль control	5.76	6.7	53,87	16.39	0.296
А	Обычные general	4.2	7.5	59,4	14.01	0.300
Б	»	5.73	13.0	47,4	10.66	0.232
[illegible]	»	5.7	30.0	59,0	14.26	0.233
	»	4.9	10.0	67,71	17.61	0.368
	»	5.7	0.0	44,4	14.06	0.215
А	Холод cold	9.75	25.0	54,7	13.75	0.311
Б	»	7.25	62.0	75,0	12.73	0.160
А	Воздействие центрифуги*	5.6	10.0	97,0	26.1	0.320
[illegible]	То же	7.7	50.0	58,0	20.02	0.333
	Нагрузка K_2HPO_4 **	7.0	57.0	26,0	5.01	0.278
Б	То же	6.6	55.0	28.0	14.20	0.356
А в целом	Обычные и стрессовые условия ***	5.6	20.0	55,6	13.04	0.287
Б в целом		6.7	40.0	51.64	11.30	0.275

*centrifugation

**K_2HPO_4

***general and stress conditions

The data confirmed our opinion that mixtures of sodium bisulfite and benzoic acid affected the animals unfavorably. The statistical differences were reliable(P about 0.05)

Under stress with K_2HPO_4 (to 1000 mg /rat/day)for the creation of renal insufficiency it was established that inorganic phosphorus preparations are slightly toxic and not good as a whole. However protein in the urine of rats in group B was only a little higher (3.3%) than group A (2.21%). (P> 0).

At the end of the chronic experiment it was important to clarify whether and in what degree the animal adapted to water soluble preservatives. In feeding benzoic acid to rats who received only benzoic acid during the entire experiment,we found that they survived doses up to 4--5 g per day (At an LD_{50} 2--2.5 g/kg body wt.). Simultaneously, in group B, despite the fact that the rats received benzoic acid during the chronic experiment mixed with sodium bisulfite the tolerance of it by the animals did not increase (P>0.1). When rats were given 2--3 ml. of bisulfite solution /day that corresponded to 0.860--1260 mg (empirical selection by us of the minimal dose producing death in rats),in group K it killed 100% of the animals, in group B,75%, in group A, 50%(in group D preservative was not given). The experimental results show that there is no adaptation to sodium bisulfite in rats. Simultaneously, group A rats appeared to be more resistant to this substance, that was confirmed by the presence in the sorbic acid of nonspecific detoxifying properties in opposition to other foreign substances entering with food products into the body. Giving rats large doses of sorbic acid (3--4 g/ rat/ day) and nisin (5--6 g/rat/day) did not lead to animals deaths and further ,did not produce any visible signs of poisoning, indicating the absense of any acute toxicity for these products.

Afeter the end of the chronic experiment, the animals were sacrificed and their internal organs examined. The most pronounced results occurred in mice. Thus, mice of group B,E, and F in comparison with mice in Group A and D had increased liver weight,enlarged spleens, ovarioes,lungs. The lowest liver weight was in group D mice. Data were statistically reliable (P~ 0.05). In rats no changes in the macro- and micro- structure of the parenchymatous organs was noted.

In relatively simple experiments one can note the following: Sorbic acid in domestic industrial production appears to be a nontoxic substance,the biological activity of this and the Czech product,despite biological admixtures is lower than that of two other products.

The polymer--one of the admixtures to the domestic sorbic acid has not been studied before us. It is a dark brown coal-like color, which pales upon exposure to light,is tasteless, has an unpleasant indeterminate odor,dissolves in HCl taking on a green color while in alkali, the color does not change. The chemical composition of the polymer is not known. The LD_{50} for mice is more than 4 g/kg body weight. Therefore the polymer appears to be only slightly toxic. When it is found in mice, there are cases of "paradoxical toxicity" properties (lethargy, rough fur etc.) and the first days of administering a small dose without such signs as during the administration of a large dose. However after 10--15 days all phenomena of toxicosis appear and these animals do not differ from the controls . As far as is known the beneficial aspects have not been studied. Possibly, this reflects on the active assimilation and adaptation by the organism of the substance.

CONCLUSIONS

!. Comparative study of the toxicity of three types of sorbic acid(Czech, domestic laboratory, and domestic industrial) showed that this preservative is nontoxic and demonstrates beneficial biological properties--increases immunological reactivity, the detoxification properties of the liver etc.

2.The polymer--an impurity in the sorbic acid in domestic industrial production-- is not toxic to mice under experimental conditions.

3.The British-manufactured nisin studied by us is definitely toxic for mice(it decreases viability during fasting and under general experimental conditions despite increased uptake of food and water and also in weight gain in males)

4.The combined effect of sorbic acid and nisin show antagonism at the expense of a nonspecific protection effect of sorbic acid; water and food uptake, body weight and viability were closer to that of controls than that of the groups of animals that received only nisin.

5. Sodium bisulfite appears to be a relatively toxic substance, especially for rats(decreases the leucocyte phagocytic activity, blood oxidase activity and the detoxification activity of the liver).

6. Benzoic acid is slightly toxic for rats and definitely toxic for mice(decreases body weight and viability under general experimental conditions and during fasting, decreases the detoxification capacity of the liver and shows evidence of carcinogenicity etc.).

7. The combined action of sodium bisulfite and benzoic acid on animals is nobeneficial, the observed indicatiors for both mice and rats being lower than controls(viability, body weight, phagocytic and oxidase activity, liver detoxification properties, carcinogenicity).

8. From the hygienic point of view, it is expeditious to limit the use of sulfer and benzoate preservatives and to expand the use of sorbic acid. Nisin plus sulfur preservatives are difinitely unsuitable for use when mixed with sorbic acid.

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Studies on Medicinal Preparations by Gas Chromatography. I

Gas Chromatography of Preservatives

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Separatory detection of preservatives by gas chromatography was examined. A mixture of sorbic acid, benzoic acid, salicylic acid, dehydroacetic acid, menadione, and methyl, ethyl, butyl, and propyl p-hydroxybenzoate was separated completely into the componental compounds without any pretreatment, under the conditions of 30% DC 550 silicone on Celite 545, at 190°, with carrier gas of hydrogen. Salicylic acid tended to undergo thermal decomposition and phenol peak sometimes appeared in the product but no such decomposition occurred in other compounds.

(Received May 30, 1963)

When analysing medicinal preparations and preservatives we are frequently confronted with extreme obstacles. Separatory detection in analysing various combination of preservatives is difficult since the same analysis method used for a single compound cannot be employed. In order to challenge this problem, there has been an active research for a long time on separatory detection of mixed preservatives. For example, the report has already been published about the detailed examimation by means of Ultra Violet spectra[1], filter paper chromatography, and paper electrophoresis methods.[2] However, these methods are not necessaril satisfactory and more time-saving and yet accurate means are still sought after.

Gas chromatography (GC) which has ostensibly marked its progress recently is thought to be the answer. In recent years, a sequence of examination result of analysis by means of GC has been reported[3,4]. However, so far, none of the report shows a satisfactory separatory detection.

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Having evaluated the GC analysis conditions, the authors have discovered the ways to separate completely the mixture into the componental compounds without any pretreatment, by using DC 550 as solid solution.

The preservatives that were detected in this experiments are nine: sorbic acid, benzoic acid, salicylic acid, dehydroacetic acid, methyl p-hydroxybenzoate, ethyl p-hydroxybenzoate, menadione, propyl p-hydroxybenzoate, butyl p-hydroxybenzoate. Figure 1 illustrates the GC of these compounds. A sharp peak is noted for each of these thus indicating a complete separation.

Here the possibility of thermal decomposition was taken into consideration.especially with SA, SOA, DHA and MNQ. First SA. SA sometimes decomposes under the GC conditions as indicated in Figure 1, and phenol peak appears in its formation (Figure 2). However, with SOA, DHA and MNQ, distillate of corresponding peak was directly collected and its IR spectra was measured. When GC infusion was compared with that of the original, perfect concurrence was noted as Figure 3 shows. Therefore, it was established that there was no decomposition by flashheater.

The above result of GC implies that it is possible to determine each preservative; hence it contributes much to the medicial preparation as well as to the analysis of preservatives in foodstuffs.

Experiment

GCG Model 2 Thermal Transmission detector was used for GC. For solid phase, infiltrated 30% of DC 550 silicone into Celite 545; particle size 80-100 meshed, 5mm X 1m of U-shaped stainless tube was filled. Aging 24° before use, 10 hours, carrier gas, H_2.

Figure 1 and 2 show the effective analysis conditions. Samples were diluted in 1-2% Me_2CO solution. It was poured by means of micro-syringe. Figure 1 shows the peak of 200-400γ.

In conclusion, our gratitude is due to Professor Kanzo Sasaki of Shionogi Laboratory for his assistance in IR spectra measurement.

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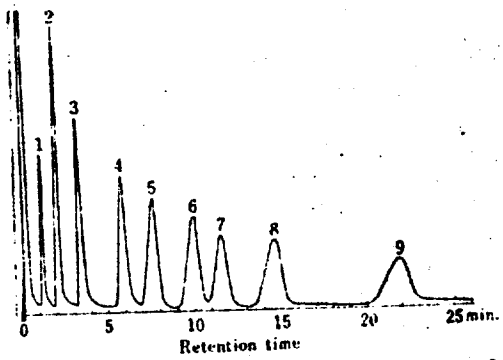

1 sorbic acid
2 benzoic acid
3 salicylic acid
4 dehydroacetic acid
5 methyl *p*-hydroxybenzoate
6 ethyl *p*-hydroxybenzoate
7 menadione
8 propyl *p*-hydroxybenzoate
9 butyl *p*-hydroxybenzoate

Conditions : column, 30% DC 550 silicone on Celite 545 1 m. × 5 mm.; temp., 190°; carrier gas, H_2 50 ml./min.; detector, T.C cell.

Fig. 1. Gas Chromatogram of a Mixture of Nine Preservatives

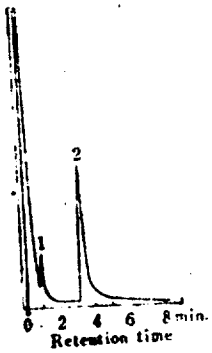

Fig. 2. Gas Chromatogram of Salicylic Acid

1 phenol (produced by thermal decomposition of 2)
2 salicylic acid

Conditions : same as Fig. 1.

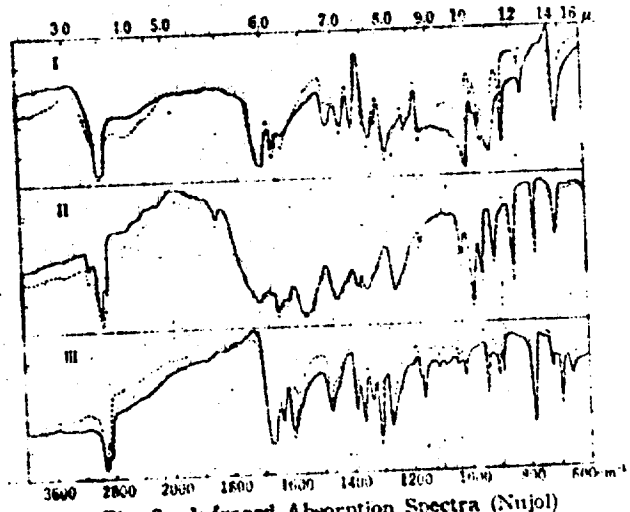

Fig. 3. Infrared Absorption Spectra (Nujol)

I sorbic acid
II dehydroacetic acid
III menadione
—— effluent
..... original

J. Nutrition 12: 365-371, 1936.

THE INFLUENCE OF CERTAIN HYDROTROPIC AND OTHER SUBSTANCES UPON FAT ABSORPTION [1, 2]

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(Received for publication June 22, 1936)

INTRODUCTION

Neuberg ('16 a, b) has shown that certain hydrotropic substances are capable of rendering soluble and diffusible, compounds, which of themselves are insoluble in water. The work of Verzar and Kuthy ('29 a, b, c, '30, '31), Verzar ('31), Wieland and Reverey ('24), Wieland and Sorge ('16) and Reinboldt ('27) has shown that bile acids combine with fatty acids, forming complex aggregations which are diffusible and stable within the reaction range prevailing in the intestine. It is quite possible that the rate of absorption of a foodstuff may depend not only on the functional activity of the digestive organs, but on the presence of hydrotropic substances in the food mixture. In this laboratory a variety of substances have been fed with fats in order to determine the effect of these substances upon the rate of fat absorption.

EXPERIMENTAL

Adult rats that had been fasted 48 hours were lightly anesthetized with ether and fed 1.5 cc. of melted fat by stomach tube. Immediately following the fat feeding 1 cc. of a water solution of the substance under investigation was fed, also by stomach tube. After a 4-hour absorption period the rat was killed and the amount of fat which had been absorbed was determined by analyzing the residues in the intestinal tract.

[1] The authors wish to express their sincere appreciation of the financial support of the Lever Brothers Company, which made this work possible.

[2] Published with the permission of the director of the Wisconsin Agricultural Experiment Station, Madison.

TABLE 1

The percentage of fat absorbed in 4 hours by rats fed 1.5 gm. of fat followed by 1 cc. of a solution containing the substance being tested

GROUP	TREATMENT	NUMBER OF ANIMALS	PERCENTAGE OF FAT ABSORBED
	Controls:		
1	Fed 1.5 cc. fat only	11	54 ± 1.6
2	Fed 1.5 cc. fat plus 1 cc. distilled water	10	55 ± 1.9
	Effect of bile. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
3	1 mg. commercial bile salts	5	53 ± 2.4
4	50 mg. commercial bile salts	5	55 ± 1.9
5	500 mg. commercial bile salts	6	19 ± 2.3
	Effect of sodium benzoate. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
6	1 mg. sodium benzoate	6	50 ± 2.0
7	50 mg. sodium benzoate	6	38 ± 2.5
8	500 mg. sodium benzoate	3	14 ± 3.3
	Effect of ethyl alcohol. Fed 1.5 cc. fat plus 1 cc. of a solution containing:		
9	5% alcohol	7	51 ± 1.7
10	15% alcohol	6	47 ± 1.8
11	40% alcohol	8	27 ± 5.8
	Effect of peptone, sucrose and a mixture of peptone and sucrose. Fed 1.5 cc. of fat plus 2 cc. of a solution containing:		
12	1 gm. peptone	5	27 ± 1.5
13	1 gm. sucrose	5	33 ± 2.4
14	1 gm. peptone and 1 gm. sucrose	6	25 ± 2.6
	Effect of KCl. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
15	0.2 mg. potassium chloride	10	48 ± 1.5
16	20.0 mg. potassium chloride	10	55 ± 1.2
17	100.0 mg. potassium chloride	7	34 ± 2.6
	Effect of $CaCl_2$. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
18	0.2 mg. calcium chloride	6	54 ± 2.5
19	20.0 mg. calcium chloride	6	54 ± 3.2
20	100.0 mg. calcium chloride	6	26 ± 2.9
	Effect of NaH_2PO_4 and glycerol. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
21	2 mg. NaH_2PO_4	6	47 ± 1.2
22	10% glycerol	7	43 ± 2.5
	Effect of sodium glycerophosphate. Fed 1.5 cc. of fat plus 1 cc. of a solution containing:		
23	2 mg. sodium glycerophosphate	7	47 ± 1.3
24	50 mg. sodium glycerophosphate	7	38 ± 2.0
25	500 mg. sodium glycerophosphate	7	20 ± 2.7

The animals were male and female rats from our stock colony and were 4 to 6 months of age. We have shown previously that there is no significance difference between the percentages of fat absorbed by male and female animals. For details of the method used and for tests dealing with the accuracy of the technic, etc., the reader is referred to the first paper of this series (Irwin, Steenbock and Templin, '36).

TABLE 2

Significance of differences in absorption

DIFFERENCE BETWEEN	MEAN DIFFERENCE	STANDARD DEVIATION OF MEAN DIFFERENCE	$\frac{MD}{\sigma MD}$
	%		
Groups (1) and (2)	1	3.8	0.3
Groups (1) and (5)	35	4.1	8.5
Groups (1) and (6)	4	4.0	1.0
Groups (1) and (7)	16	4.4	3.6
Groups (1) and (8)	40	5.5	7.3
Groups (1) and (9)	3	3.5	0.9
Groups (1) and (10)	7	3.6	1.9
Groups (1) and (11)	27	8.8	3.1
Groups (1) and (12)	27	3.3	8.2
Groups (1) and (13)	21	4.2	5.0
Groups (1) and (14)	29	4.6	6.3
Groups (1) and (15)	6	3.3	1.8
Groups (1) and (16)	1	3.0	0.3
Groups (1) and (17)	20	4.5	4.4
Groups (1) and (20)	28	4.8	5.8
Groups (1) and (21)	7	3.0	2.3
Groups (1) and (22)	11	4.2	2.6
Groups (1) and (23)	7	3.0	2.3
Groups (1) and (24)	16	3.7	4.3
Groups (1) and (25)	25	4.4	5.7

The base fat fed was a commercially hydrogenated vegetable shortening (fat A)[3] which was chosen because of its uniform composition and stability. Data showing the percentages of fat absorbed in 4 hours by adult rats fed 1.5 cc. of the fat followed by 1 cc. of a solution of the various substances tested are given in table 1.

Table 2 records the statistical significance of the differences in the means of the several groups.

[3] Fat A was a commercial product manufactured by hydrogenating cottonseed oil to an iodine number of approximately 70. The saponification number was 196.0.

DISCUSSION

Bile salts. Verzar and Kuthy ('29 b) have advanced the theory that the bile acids aid fat absorption by combining with the fatty acids, 'carrying' them through the intestinal wall. In a more recent publication Verzar and Laszt ('34) report an experiment in which they anesthetized rats with urethane, ligated a loop of the intestine—leaving the circulation intact, and after washing out the loop with physiological saline, injected into it a mixture of oleic acid and bile acid. After a definite time interval the amount of fatty acid absorbed was determined. They found that the absorption of oleic acid was definitely increased by glycocholic acid under the conditions of their experiment. In our experiments the addition of 1 or 50 mg. of commercial bile salts[4] did not increase the rate of absorption of fats. The addition of 500 mg. decreased absorption, although this figure (19%, table 1) is not a reliable one due to the laxative effect of this quantity of bile salts. No indications of diarrhea were observed in our animals when 1 or 50 mg. of bile salts were fed. In Verzar's experiment normal secretions into the intestinal tract were in part excluded; in ours, the digestive tract remained intact during the absorption period with the secretions and movements of the intestine functioning normally. Possibly in our experiments the optimum amount of bile acids was secreted by the liver, and, therefore, the further addition of small amounts of bile salts had no effect.

Sodium benzoate. Sodium benzoate is one of the many organic salts which shows hydrotropic properties. When fed with fat, 1 mg. did not affect the rate of absorption, but 50 or 500 mg. definitely lowered it (table 1).

Alcohol. Cori, Villiaume and Cori ('30) observed that small quantities of ethyl alcohol did not alter the rate of absorption of sugar. We obtained similar results with fat; a small quantity of alcohol had no marked effect on fat absorption, although 1 cc. of a 40% alcohol solution decreased it from 54% to 27% (table 1).

[4] Supplied through the kindness of the Wilson Laboratories. The preparation consisted predominately of crude sodium glycocholate and sodium taurocholate.

Peptone and sucrose. Since, in the ordinary diet, fat is usually absorbed in the presence of protein and carbohydrate, fat was fed with peptone, with sucrose, and with a mixture of the two. From the data presented in table 1 it is clear that fat was absorbed less rapidly when fed with these foodstuffs than when fed alone.

Calcium and potassium. The effect of electrolytes on absorption is, as yet, an unsettled question. Magee and Reid ('27), Magee and Southgate ('29), and Magee and Sen ('31, '32) have observed that small amounts of $CaCl_2$ in the lumen of the rabbit's intestine increased its tone and stimulated contraction. Glucose diffused more rapidly from sections of the surviving intestine into a Tyrode solution containing calcium than it did into a calcium-free solution. Magee and co-workers found also that thyroparathyroidectomized rats with a low blood calcium did not absorb as much glucose as normal rats and that the addition of calcium to the sugar solution increased absorption.

Contrary evidence has been presented by Gellhorn and Skupa ('33) who perfused the gut of frogs with a glucose solution and the blood vessels with a modified Ringer's solution. By the addition or omission of one salt or another these investigators found that when both calcium and potassium were omitted from the perfusiate, permeability of the gut to glucose was increased. The addition of $CaCl_2$ alone to such a solution decreased permeability, but when as little as 0.0075% of KCl was added, the permeability of the gut to glucose was increased.

Whether or not the action of calcium and potassium ions is specific for glucose absorption or is fundamental to the process as a whole cannot be determined until more evidence is presented. Under the conditions of our experiment small quantities of $CaCl_2$ or KCl had no effect on fat absorption; larger amounts of either of these salts depressed absorption (table 1).

Phosphates. Verzar and Laszt ('34) obtained a definite increase in the absorption of oleic acid when glycerophosphate was injected into ligated loops of rat's intestine along with oleic acid and bile acids. Sinclair ('29), Verzar and Laszt ('34), and also Süllmann and Wilbrandt ('34) believe that the transformation of absorbed fatty acids into phospholipids is an essential step in the resynthesis of neutral fat in the intestinal mucosa. Additional evidence supporting this idea is to be found in the work of Smedley MacLean and Hoffert ('23) who found that yeast grown in an oxygenated medium containing phosphates stored much more fat than when grown on a phosphate-free medium. Magee and Reid ('31) reported that the presence of 0.2% NaH_2PO_4 in a 0.75 M glucose solution increased its rate of absorption by 50%. This work has been confirmed by Wilbrandt and Laszt ('33), and it has been suggested that the formation of glycerophosphoric acid is a step in the absorption of glucose. In our experiments NaH_2PO_4, glycerol, and sodium glycerophosphate solutions were fed along with fat, and the absorption of the fat determined. In table 1 it can be seen that the presence of any one of these substances in the intestinal tract during digestion and absorption of fat retarded rather than stimulated the process.

SUMMARY

The influence of water, bile salts, sodium benzoate, ethyl alcohol, peptone, sucrose, KCl, $CaCl_2$, NaH_2PO_4, glycerol and sodium glycerophosphate on the rate of absorption of fats has been determined. In general the feeding of small amounts of any one of these substances had little or no effect, but larger amounts invariably decreased the rate of fat absorption.

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CCLV. STUDIES IN FAT METABOLISM.

I. THE OXIDATION OF BUTYRIC, CROTONIC AND β-HYDROXYBUTYRIC ACIDS IN PRESENCE OF GUINEA-PIG LIVER SLICES.

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(*Received July 11th, 1935.*)

UNTIL lately the study of the metabolism of fatty acids has depended on methods with which it is difficult to obtain quantitative results and comparisons.

Recently, however, Quastel and Wheatley [1933] have found that fatty acids are oxidised at considerable rates by slices of liver *in vitro* and give as one of their oxidation products acetoacetic acid, as in the body. Modern micromanometric technique can, therefore, now be applied to problems of fatty acid metabolism.

In the present work a study has been made of the kinetics of the oxidation of butyric, crotonic and β-hydroxybutyric acids by the liver of the guinea-pig, and this has led to a partial elucidation of the mechanisms by which these substances are oxidised to acetoacetic acid.

EXPERIMENTAL METHODS.

The manometric methods of Warburg [1926] have been used throughout. Measurements of respiration have been made by immersing tissue slices in a medium containing sodium β-glycerophosphate as buffer, 0·2 ml. of 6% KOH being present in a side-tube of the manometric vessel. Estimations of acetoacetic acid have been made in the solutions after removal of the tissue slices. The experimental period has usually been two hours at 37°. The mean rates for respiration refer to the period ¼ to 2 hours and those for acetoacetic acid formation to the period 0 to 2 hours.

In accordance with the notation introduced by Warburg, the respiration (Q_{O_2}) is defined as the number of μl. of oxygen (reduced to N.T.P.) absorbed per mg. dry weight of tissue per hour, and the rate of production of acetoacetic acid (Q_{Ac}) is similarly defined in terms of gas evolution. Our manometric method of estimating acetoacetic acid gives an evolution of one molecule of carbon dioxide per molecule of acetoacetic acid. Hence Q_{Ac} can be regarded as measured in terms of volumes of CO_2, or, according to the usual convention, as measured in terms of the volume which acetoacetic acid would occupy if it behaved as a perfect gas at N.T.P.

Media. All solutions have been so made up as to have an osmotic pressure approximately equal to that of 0·16 *M* NaCl. Uni-univalent salts employed are made up in stock solutions to 0·16 *M*, or to submultiples of this strength, dilutions being made with 0·16 *M* NaCl. Salts of other valency-types are made up to the appropriate strengths, *e.g.* sodium fumarate to 0·107 *M*. With solutions so

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made up, the medium in which the tissue is immersed can readily be made isotonic by mixing the various stock solutions.

The volume of medium used in each vessel has been usually 3·2 ml., this choice of volume being convenient for performing dilutions. The volume is made up to 3·2 ml. with 0·16 *M* NaCl after all other desired substances are present.

The composition of a typical medium is as follows:

Sodium glycerophosphate	0·067 *M*
KCl	0·002 *M*
$CaCl_2$	0·001 *M*
Sodium butyrate	0·01 *M*
NaCl	0·051 *M*

In all media employed, unless otherwise stated, the concentration of K^+ is 0·002 *M*, and that of Ca^{++} 0·001 *M*.

Glycerophosphate buffer. Quastel and Wheatley [1933] found that phosphate inhibited partially the oxidation of butyric acid to acetoacetic acid by liver. Phosphate buffer solutions precipitate calcium ion and cannot be used in a medium of the Locke type. Following Quastel and Wheatley, we have therefore adopted sodium β-glycerophosphate as a buffer. The salt is brought to p_H 7·4 with HCl, and a 0·105 *M* solution is used, of which usually 1·9 ml. are present in a total volume of 3·2 ml.

According to Meyerhof and Kiessling [1933] $p_{K'_2}$ for β-glycerophosphoric acid is about 6·33; hence at p_H 7·4 the buffering action is weak, particularly in the direction of rising p_H. Quastel and Wheatley [1934] reported a drop in p_H of about 0·2 during experiments of 3 hours' duration with guinea-pig and rat liver slices. Glycerophosphate solutions undergo hydrolysis on keeping, with liberation of phosphate and fall of p_H. They may however be kept several days at 0° without much change.

Tissue slices. It has been found that the accuracy of the experiments depends mainly on the use of slices of similar thickness throughout any one experiment. The thickness of slices used varies from one experiment to another, but has usually lain between the limits 0·25–0·45 mm. Several slices are commonly employed in each vessel, since the total dry weight desired has usually been 15–25 mg. The use of several slices appears to help to smooth out differences of behaviour.

Livers. The livers of young guinea-pigs, mainly of weights 270–330 g., fed on bran, oats and greens, have been employed. The animals were bled from the throat before removing the livers, the blood of which is therefore partially removed.

Neutralisation of acids. All fatty and other acids are neutralised before use, solutions of the sodium salts being prepared.

Manometric estimation of acetoacetic acid. As was found by Quastel and Wheatley [1933], acetoacetic acid can be estimated manometrically by measurement of the volume of gas produced in its breakdown to acetone and carbon dioxide, aniline being used as a catalyst for the process.

Acetoacetic acid breaks down spontaneously in solution, giving the same end-products except in very alkaline solutions [Widmark, 1920]. The rate of breakdown is slow in neutral solutions but becomes faster in acid solutions. Below are given some data on this point calculated from the measurements of Widmark.

Values of the rate of decomposition have also been measured by us manometrically, and are summarised in the last column of the table. The agreement with the figures of Widmark is quite satisfactory.

Decomposition of acetoacetic acid at 37°.

k_{-1} (min.) (W.)	p_H	% decomposition In 15 min.	% decomposition In 2 hours	k_{ent} (J. and Q.)
$4{\cdot}5\ .10^{-3}$	<2·0	6·5	41·7	$4{\cdot}8.10^{-3}$
$1{\cdot}31.10^{-3}$	4·0	1·9	14·6	—
$5{\cdot}8\ .10^{-4}$	4·5	0·9	6·7	$5{\cdot}5.10^{-4}$
$2{\cdot}5\ .10^{-4}$	5·0	0·4	3·0	—
$8{\cdot}0\ .10^{-5}$	7–8	0·1	1·0	(2.10^{-4})

The table shows that acetoacetate formed in neutral solutions, as in our experiments with tissues, will not be appreciably decomposed during experiments of two hours' duration, by the homogeneous process here considered.

For the estimation of acetoacetic acid, the neutral buffered solutions used in tissue experiments must be acidified to a p_H of 5 or below before adding the aniline catalyst in order to avoid retention of carbon dioxide by the medium. The table shows that the solution can be kept at 37° for 15 min. at p_H 4·5 with less than 1 % loss of acetoacetic acid.

We have therefore usually employed the following technique. At the end of the experiment, the tissue slices are removed from the manometer vessels. The residual solution, usually 3·2 ml., is at once acidified by adding 0·3 ml. of a suitable solution of acetic acid or acetic-acetate buffer, which brings it to a p_H near 4·5 (B.D.H. "4·5" indicator may be used). 0·2 ml. of a solution of aniline hydrochloride containing 0·09 g. of the salt is run into a side-tube of the vessel[1]. The vessel, attached to its manometer with air as gas phase, is then shaken for 15 min. in the thermostat at 37° before the manometer is read and the aniline salt is added to the solution containing acetoacetic acid. The acetoacetic acid is now decomposed according to a unimolecular law, the precise rate depending on the buffering of the solution, which determines the p_H it assumes on addition of the more acid aniline hydrochloride solution. According to Widmark and Jeppsson [1922] the optimum p_H for the reaction is about 4·1, but we have not troubled to choose conditions which are exactly optimum. Under our conditions the acetoacetic acid is half decomposed in 5–6 min. and after 50 min. evolution of gas may be considered complete.

Addition of the concentrated aniline hydrochloride to solutions containing no acetoacetic acid results in evolution of gas (some 8 mm. of Brodie's fluid or 12μl. of gas under the conditions described), presumably due to expulsion of air and a "blank" correction, which need not be determined in every experiment, must be applied.

In tissue experiments where no acetoacetic acid was expected to be formed, the pressure changes obtained when corrected for "the blank" have sometimes given slightly negative values. This absorption is probably due to a very small oxygen uptake by tissue débris or extract, which continues after addition of aniline. Its magnitude has always been less than about 8 μl. and it has been neglected.

The CO_2 outputs from sodium acetoacetate solutions made by hydrolysing known amounts of ethyl acetoacetate have been measured and values up to 97–98 % of the theoretical have been obtained. Since the acetoacetate solutions are unstable even when kept at 0°, we take this to indicate a real and exact equivalence between CO_2 output and acetoacetic acid.

[1] If a side-tube of the vessel previously contained alkali (to absorb CO_2), the alkali is removed with filter-paper, and the side-tube rinsed with dilute acid and roughly dried, employing filter-paper again.

The method is suitable for measuring quantities of acetoacetic acid which give 100 μl. of CO_2 and upwards. For 10–20 μl. the accuracy is doubtful, but the errors will probably be of a similar order in estimations made in any one experiment.

A question to be considered is whether for the purposes of this work the manometric method is specific for acetoacetic acid, or whether other substances, and in particular other β-keto-acids, will react similarly with aniline.

Oxaloacetic acid reacts with aniline, but much more rapidly [Ostern, 1933], so that if quantities appreciable in relation to acetoacetic acid were present they could be detected by a rapid fall in the unimolecular constant of the reaction. No such indications of the formation of oxaloacetic acid have been found in this work.

This work, together with that of Quastel and Wheatley [1933; 1934], is apparently the first in which the formation of acetoacetic acid from fatty acids by the liver has been measured as such. Embden and Marx [1908] distilled their solutions after each experiment and obtained in the distillate an iodoform-yielding substance, presumed to be acetone and in some cases identified as acetone. Embden and Engel [1908] showed in some cases that the iodoform-yielding substance was preformed in the solutions only to a small extent and was mainly formed on heating. There was thus a strong presumption that acetoacetic acid is the product chiefly in question, but conceivably, as Hurtley pointed out [1916], it might be acetonedicarboxylic acid.

Respiration and acetoacetic acid production.

In Fig. 1 are given curves showing the manner in which the rate of acetoacetic acid production (Q_{Ac}) by liver slices varies with the concentration of the substrate in the medium. In the absence of added substrate, Q_{Ac} has a small but

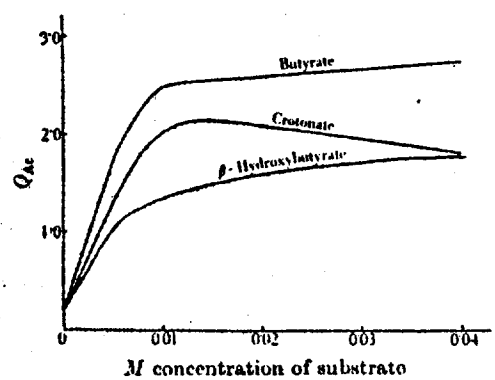

Fig. 1. Rates of acetoacetic acid production by guinea-pig liver. [Oxygen; glycerophosphate buffer.]

definite value. At low concentrations of fatty acid, Q_{Ac} increases approximately proportionally to the concentration. With butyric acid, Q_{Ac} reaches almost its highest value at a concentration of 0·01 M, with β-hydroxybutyric acid the saturation concentration is more nearly 0·03 or 0·04 M. With crotonic acid, however, Q_{Ac} passes through a maximum at a concentration of about 0·015–0·02 M. The curves are smoothed curves drawn by combining the results of several experiments.

The behaviour of butyric and β-hydroxybutyric acids is normal, whilst it appears that crotonic acid in some manner inhibits its own oxidation at high concentrations.

Influence of ionic environment on acetoacetic acid production.

The rate of production of acetoacetic acid is also influenced by the concentration of potassium and calcium ions in the medium, at least in the case of butyric and crotonic acids. Experiments are given in Table I showing the effect of varying (*a*) the potassium ion concentration, (*b*) the calcium ion concentration and (*c*) both potassium and calcium ion concentrations.

Table I. *Effect of varying potassium and calcium ion concentrations.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Exp.	Substrate (M)	Concentrations (M) [K+]	[Ca++]	Q_{O_2}	Q_{Ac}
1	Butyrate 0·01	0	0·001	7·43	2·52
		0·001	0·001	8·37	2·98
		0·002	0·001	9·13	3·10
		0·005	0·001	9·20	2·99
2	Butyrate 0·01	0·002	0	8·56	1·98
		0·002	0·001	8·70	2·93
		0·002	0·002	8·01	2·50
		0·002	0·004	7·86	2·54
3	Butyrate 0·01	0	0	7·24	1·64
		0·001	0·0005	9·50	3·32
		0·002	0·001	9·13	3·20
		0·005	0·0025	8·85	2·71
4	Crotonate 0·02	0·002	0	7·37	1·69
		0·002	0·0005	7·89	2·28
		0·002	0·001	7·92	2·35
		0·002	0·002	7·44	2·22
5	Crotonate 0·01	0	0	5·88	1·83
		0·0005	0·00025	7·57	2·34
		0·002	0·001	7·77	2·58
6	β-Hydroxybutyrate 0·01	0	0	4·25	1·46
		0·0006	0·0003	5·03	1·20
		0·002	0·001	4·89	1·39

The experiments show that with crotonic and butyric acids Q_{Ac} has optimum values when [K+] is about 0·002M and [Ca++] is about 0·001M. These concentrations of potassium and calcium ions have therefore been maintained in the media throughout the rest of this work.

On the other hand, the production of acetoacetic acid from β-hydroxybutyric acid is not definitely influenced by the ionic environment.

It has been found that the rate of acetoacetic acid production from butyrate is approximately the same in a glycerophosphate medium as in Ringer's solution, when the initial p_H of both media is 7·4. It is therefore unlikely that β-glycerophosphate has any great specific action on the oxidation of fatty acids.

Effect of p_H variation on acetoacetic acid formation.

The effect of varying the p_H of the medium is shown in Table II, where in the several vessels employed in each experiment the same mixture of O_2 and CO_2 is present, and the bicarbonate concentration is varied. Bicarbonate-CO_2 here replaces glycerophosphate as buffer. The experiments show that, with all three

Table II. *Effect of* p_H *on rate of production of acetoacetic acid by guinea-pig liver.*

O_2+CO_2. p_H varied by variation of bicarbonate concentration and calculated roughly to midpoint of experiment by assuming $Q_{CO_2}=3{\cdot}5$.

Exp.	Substrate	Initial % CO_2	Relative values for Q_{Ac} at p_H 6·8	7·1	7·4	7·7	8·0
1	Butyrate 0·01 *M*	5	60	84	100	97	—
2	Butyrate 0·01 *M*	1·3	—	89	100	87	70
3	β-Hydroxybutyrate 0·01 *M*	5	54	72	100	78	—
4	β-Hydroxybutyrate 0·02 *M*	1·5	—	89	94	100	95
5	Crotonate 0·02 *M*	5	57	83	81	100	—
6	Crotonate 0·02 *M*	5	58	70	91	100	—
7	Crotonate 0·02 *M*	1·3	—	65	89	100	95
8	Crotonate 0·02 *M*	1·3	—	72	85	100	98

Hence approximate optimum p_H values are: butyrate 7·4–7·6; β-hydroxybutyrate 7·4–7·7; crotonate 7·7–7·9.

acids, the rate of formation of acetoacetic acid varies considerably with the p_H, the variation being of the same order as is shown in glycolysis [Warburg *et al.*, 1924].

In the case of butyrate and β-hydroxybutyrate, Q_{Ac} is maximum at about the physiological p_H, whilst in the case of crotonate the optimum is displaced definitely to the alkaline side.

Mean rates of acetoacetic acid formation.

In Table III are assembled data showing the mean rates of acetoacetic acid formation from the three acids. It is evident that the highest rate is given by butyric acid, followed closely by crotonic acid, whilst the rate from β-hydroxybutyric acid is appreciably lower. The rate of acetoacetic acid formation by the

Table III. *Mean rates of acetoacetic acid formation.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Substrate	Conc. (*M*)	No. of experiments	Range of values of Q_{Ac}	Mean Q_{Ac}	Average deviation from mean
—	—	42	0·00–0·68	0·20	±0·07
Butyrate	0·01	39	1·09–4·44	2·55	±0·46
Crotonate	0·01	14	0·79–3·02	2·21	±0·53
Crotonate	0·02	21	1·27–3·25	2·41	±0·36
dl-β-Hydroxybutyrate	0·01	10	1·15–1·99	1·43	±0·17
dl-β-Hydroxybutyrate	0·02	6	1·53–2·44	1·91	±0·27
Animal starved 24 hours before killing.					
Butyrate	0·01	7	1·48–3·92	3·05	±0·48

Effect of size of liver on metabolism.

Wt. liver as % wt. animal	No. of experiments	Range of values	Mean values	Average deviation from mean
Q_{Ac} from butyrate 0·01 *M*.				
2·6–3·6	12	1·97–3·67	2·61	±0·35
3·7–4·6	15	1·85–4·44	2·74	±0·45
4·7–6·9	11	1·09–2·82	2·14	±0·41
Q_{O_2} without added substrates.				
2·8–3·6	13	3·67–5·93	4·84	±0·62
3·7–4·4	20	4·21–5·52	4·96	±0·35
4·5–7·2	20	3·85–6·84	5·39	±0·75

liver in absence of added fatty acid is low, too low for accurate measurement, and can for most purposes be neglected in considering the acetoacetic acid formation in presence of added fatty acid.

Q_{Ac} varies considerably from one liver to another. Experiments seem to indicate that the state of nutrition plays a part, for Q_{Ac} in the case of butyrate as substrate appears to be higher when the animal has been starved for 24 hours before being killed. This type of effect, which requires further investigation, may throw light on the question whether fatty acid substrates compete with carbohydrates for oxidation, or whether, as is sometimes suggested, "fats burn in the fire of the carbohydrates".

The size of the liver in young guinea-pigs is very variable. In considering this matter, we use the weight of the liver (wet weight) expressed as a percentage of the weight of the animal. The figures for 120 animals show a variation of this percentage between 2·6 and 7·2; 50% of the figures lie between 3·6 and 4·5, 75% between 3·3 and 5·1, and 90% between 3·1 and 5·6. The data given in Table III show that the weight of the liver is a factor in determining Q_{Ac}. In the case of butyrate as substrate heavy livers give lower values of Q_{Ac}. The same is true for crotonate, for which the data are not given.

Data on the respiration of liver in absence of added substrates have been calculated (Table III), which show that Q_{O_2} does not vary very significantly with the weight of the liver. The tendency is for larger livers to give higher values for Q_{O_2}, but the most definite finding is that the respiration of medium-sized livers is less variable than that of light or heavy livers. Further investigation is desirable to show whether or not the percentage of fat in the liver, which may influence considerably the dry weight (used in calculating metabolic quotients), varies with the size of the liver.

Correlation between acetoacetic acid production and increased respiration.

It is shown in Table IV that, when butyrate or crotonate is present in the medium, the respiration of the tissue is raised. There is found to be a very close correlation between the increase in the rate of production of acetoacetic acid, *i.e.* Q_{Ac}, and the increase in respiration, Q_{O_2}. The ratio between the two increases

Table IV. *Acetoacetic acid formation and oxygen consumption.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Exp.	Without substrate Q_{O_2}	Without substrate Q_{Ac}	Substrate	With substrate Q_{O_2}	With substrate Q_{Ac}	Extra Q_{Ac} / Extra Q_{O_2}
1	4·12	0·12	Butyrate 0·01 *M*	6·91	1·69	0·56
2	4·14	0·14	"	7·10	2·07	0·65
3	4·66	0·18	"	8·18	2·27	0·60
4	4·37	0·25	"	8·32	2·71	0·62
5	4·53	0·20	"	8·68	2·95	0·67
6	4·93	0·24	"	9·25	3·05	0·65
7	4·66	0·19	"	9·29	3·39	0·69
8	4·43	0·23	Crotonate 0·01 *M*	6·32	1·58	0·71
9	4·19	0·34	"	7·22	2·41	0·68
10	5·12	0·18	"	8·17	2·64	0·81
11	4·75	0·08	"	8·41	2·65	0·70
12	4·33	0·13	"	7·67	2·67	0·76
13	5·39	0·25	β-Hydroxybutyrate 0·01 *M*	6·11	1·36	1·5
14	4·66	0·19	"	5·13	1·46	(2·7)
15	5·79	0·08	"	6·84	1·99	1·25
16	4·93	0·24	β-Hydroxybutyrate 0·02 *M*	6·88	1·94	0·87
17	5·79	0·68	"	7·45	2·44	1·06

is constant almost within the experimental error. Under the given conditions the ratio has the mean value 0·63 for butyrate and 0·73 for crotonate. Possibly also in the case of β-hydroxybutyrate the variation does not exceed experimental error.

The value of the ratio, however, depends on the concentration of the substrate (Table V). It is noteworthy that on continuing to increase the substrate concentration beyond the point at which Q_{Ac} has reached its highest value, the respiration continues to rise. There is an indication here that the oxidation of the substrates follows two different paths, one to acetoacetic acid and another to some other product or products.

Table V. *Acetoacetic acid formation and oxygen consumption.*

Guinea-pig liver. Glycerophosphate buffer. Oxygen.

Exp.	Substrate (M)	Q_{O_2}	Q_{Ac}	Extra Q_{Ac} / Extra Q_{O_2}
1	—	4·33	0·13	—
	Crotonate 0·005	7·26	1·66	0·52
	" 0·01	7·67	2·67	0·76
	" 0·02	8·53	2·83	0·64
	" 0·04	8·64	2·38	0·52
2	Butyrate 0·0025	8·34	0·81	—
	" 0·005	9·22	1·68	—
	" 0·01	10·1	2·49	—
	" 0·02	10·7	2·60	—

Relative rates of acetoacetic acid production from butyric and crotonic acids.

A series of experiments has been made in which the actions of each liver on butyric and crotonic acids have been compared (Table VI). It will be seen that a close parallelism exists between the values of Q_{Ac} observed for the two acids. A liver that gives a low value with one acid gives a low value for the other. Crotonic

Table VI. *Comparative rates of acetoacetic acid formation from butyric and crotonic acids.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.
Butyrate when present is at a concentration of 0·01 M.

Exp.	Conc. of crotonate when present (M)	Q_{Ac} in presence of Crotonate	Q_{Ac} in presence of Butyrate	Ratio	Mean ratio
1	0·01	0·79	1·09	0·72	
2	"	1·37	1·67	0·82	
3	"	1·57	2·01	0·78	
4	"	2·10	2·77	0·76	
5	"	2·20	2·32	0·95	
6	"	2·62	3·12	0·84	0·81
7	0·015	1·72	1·85	0·93	
8	"	1·91	2·24	0·85	
9	"	2·12	2·32	0·91	0·90
10	0·02	1·96	2·02	0·97	
11	"	2·23	2·43	0·92	
12	"	2·33	2·50	0·93	
13	"	2·34	2·77	0·84	
14	"	2·36	2·45	0·96	
15	"	2·43	2·73	0·89	
16	"	2·46	2·81	0·88	
17	"	2·87	3·27	0·88	
18	"	3·25	3·67	0·89	0·91

acid gives lower values for Q_{Ac} than does butyric acid, and at a given concentration the ratio of the values of Q_{Ac} is almost constant, having a maximum value (0·9) at the concentration of crotonate which gives maximum values of Q_{Ac} (0·015–0·02 M, Fig. 1). This close parallelism between the values of Q_{Ac} is a strong indication of some similarity in the mechanism of oxidation of the two acids.

Competition between substrates.

Results are given in Table VII of some experiments on the respiration and acetoacetic production when two of the acids are present together.

When β-hydroxybutyrate and butyrate are present together, the rates of production of acetoacetic acid from the two substances are not additive. Yet

Table VII. *Competition between the acids as substrates.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Exp.	Concentration (M) in medium of: Butyrate	Crotonate	β-Hydroxybutyrate	Q_{O_2}	Q_{Ac}
1	—	—	—	4·66	0·19
	—	—	0·01	5·13	1·46
	0·01	—	—	9·29	3·39
	0·01	—	0·01	9·52	3·93
2	—	—	—	4·93	0·24
	—	—	0·02	6·88	1·94
	0·01	—	—	9·25	3·05
	0·01	—	0·02	9·57	3·59
3	0·01	—	—	11·4	4·44
	0·01	0·01	—	11·0	3·82
	0·01	0·02	—	11·5	3·59
	0·01	0·04	—	10·8	2·71
4	—	—	—	4·19	0·34
	0·01	—	—	8·45	2·80
	—	0·01	—	7·22	2·41
	0·01	0·01	—	7·58	2·35

although Q_{Ac} from butyrate is approximately maximum, the value of Q_{Ac} can be raised by the presence of β-hydroxybutyrate. It is evident, therefore, that the production of acetoacetic acid from these two substances may proceed, at least partially, by quite independent paths.

On the other hand, when crotonate is added to butyrate, Q_{Ac} is lowered. The experiments agree with the view that, when both acids are present, crotonate exerts on the oxidation of butyrate the same inhibitory action that it exerts on its own oxidation at sufficiently high concentrations. There is no evidence that the acids are oxidised to acetoacetic acid by independent paths.

It may be mentioned here that under anaerobic conditions guinea-pig liver does not oxidise butyric, crotonic or β-hydroxybutyric acid appreciably to acetoacetic acid. This was shown by experiments similar to those already recorded, except that nitrogen replaced oxygen in the gas phase. The highest value of Q_{Ac} found was 0·14, which does not exceed the experimental error.

The action of inhibitors.

The action of a number of substances likely to inhibit the oxidation of one or more of the fatty acids was investigated largely with a view to finding differential effects on one or more of the acids, which might give information regarding the mechanisms of the oxidations.

It will be seen (Table VIII) that among the substances examined are several unsaturated compounds, which, it was thought, might inhibit particularly the oxidation of crotonic acid. This however was not found to be the case, although there is one possible exception.

With all substances examined which cause much inhibition of acetoacetic acid formation, the effects on crotonic acid and butyric acid are very similar, with the qualification that as a rule the inhibition is slightly greater for crotonic acid (Table VIII).

The inhibitory action of propionate on oxidation of butyrate had already been found by Quastel and Wheatley [1933]. It is now found that oxidation of crotonate is inhibited to a similar extent, whilst that of β-hydroxybutyrate is only very slightly lowered. Propionate is itself oxidised by the liver without acetoacetic acid formation.

Acrylate[1] exerts an inhibitory action on acetoacetic acid formation similar to, but weaker than, that of propionate and like propionate increases the respiration of guinea-pig liver, but to a smaller extent.

Allyl alcohol, already known as a powerful respiratory poison, inhibits the respiration of liver strongly, its action being progressive with time. It lowers Q_{Ac} in presence of crotonate and butyrate still more strongly, inhibition being still in evidence at a concentration of 0·0003 M. Observations suggest that at low concentrations the alcohol is strongly taken up by the tissue, since the inhibition seems to depend on the weight of tissue present. Allyl alcohol is not therefore convenient for quantitative experiments.

Several powerful inhibitors have been found in the aromatic series. Cinnamic, β-phenylpropionic and benzoic acids all cause considerable inhibitions of acetoacetic acid formation from butyric and crotonic acids at concentrations of 0·0005 M, the inhibition of oxidation of crotonic acid being greater than of butyric acid. The oxidation of β-hydroxybutyric acid is much less inhibited. The aromatic acids mentioned above do not diminish the respiration of the liver in presence of the fatty acids to a greater extent than would be expected from the diminution in production of acetoacetic acid, *i.e.* on the basis of the relation between respiration and acetoacetic acid production which has already been found (Table IV). The diminution in respiration is in fact usually less than would be expected from the ratios previously given. As far as our experiments go, they indicate that weak solutions of the aromatic acids in question affect no other respiratory processes in the liver than the oxidation of fatty acids to acetoacetic acid.

Our experiments give no indication that these aromatic acids are themselves undergoing appreciable destruction in the liver, as according to the work of Knoop and Dakin they do in the intact organism. If the acids disappeared during our experiments, it might be expected that their inhibitory effect on respiration (due to their inhibitory effect on fatty acid oxidation) would be lessened, and an increase would take place in the respiration relative to the "control". No such phenomenon was observed. It was also observed in one experiment (Table VIII) that the inhibitory effect of benzoate on acetoacetic acid production is independent of the weight of tissue present, which indicates that benzoate is neither very greatly adsorbed nor destroyed by quantities of tissue such as we employ in our work.

Tropic (α-phenyl-β-hydroxypropionic) acid is much less toxic to acetoacetic acid production than are cinnamic and β-phenylpropionic acids.

Fluoride is found to be a strong inhibitor of acetoacetic acid formation from

[1] Not a pure preparation.

Table VIII. *Action of inhibitors.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

	% decrease in Q_{Ac} due to inhibitor Substrate (0·01 *M*)		
	Butyrate	Crotonate	β-Hydroxybutyrate
Effect of propionate (0·01 *M*)			
Exp. 1	47	—	9
" 2	—	63	6
" 3	71	74	—
Effect of acrylate (0·02 *M*)	43	57	6

	0·02 *M* crotonate			
Allyl alcohol (*M*) ...	0·04	0·006	0·001	0·0003
Effect of allyl alcohol				
% decrease in Q_{O_2}	80	75	59	3
% decrease in Q_{Ac}	100	100	94	23

		% decrease in Q_{Ac} Substrate (0·01 *M*)		
	Conc. (*M*)	Butyrate	Crotonate	β-Hydroxybutyrate
Effect of cinnamate				
	0·01	—	84	—
	0·005	72	83	23
	0·002	71	87	—
	0·0005	63	88	11
	0·0001	18	32	(− 9)
Effect of β-phenylpropionate				
	0·0005	54	70	—
Effect of benzoate				
	0·01	—	90	—
	0·0002	—	78	—
	0·001	40	63	
	0·0005	—	39	—
Effect of tropate				
	0·02	22	—	10
	0·005	9	—	25

		% decrease in Q_{Ac}	
		Crotonate	Butyrate
Effect of fluoride			
	0·02*	90	—
	0·01	82	—
	0·005	75	—
	0·0031	—	70
	0·003	68	—
	0·0025	75	79, 66
	0·0015	31	—
	0·00075	18	—

		% effect on Q_{Ac}	
		Butyrate	Crotonate
Effect of fumarate			
	0·02–0·05	+12	− 9
		+ 4	−18
		+ 2	− 8

* In this solution calcium fluoride precipitated at room temperature, which was not observed in weaker solutions.

Table VIII (*cont.*).

Inhibition in relation to weight of tissue slices.

Dry wt. tissue mg.	Conc. of benzoate M	Q_{Ac} in presence of butyrate	% inhibition
28·9	0	1·69	—
15·6	0·002	1·13	33
19·8	0·002	1·12	34
44·2	0·002	1·10	35

crotonic and butyric acids. The effect persists down to concentrations of 0·001 M. The stronger solutions of fluoride have also a considerable and progressive inhibitory action on respiration, but weaker solutions (say 0·0025 M) inhibit respiration no more than would be expected from their effect in diminishing oxidation of the fatty acids. The action of fluoride in weak solutions is probably therefore fairly specific. The inhibitory action on acetoacetic acid formation is exerted at concentrations similar to those at which fluoride inhibits glycolysis [Dickens and Šimer, 1929].

A slight differential effect on the production of acetoacetic acid from butyric acid and crotonic acid is shown by strong solutions of fumarate. Fumarate slightly increases Q_{Ac} in presence of butyrate and decreases it slightly in presence of crotonate. This is the only case we have observed where a substance affects the rates in opposite directions, but the effect does not lie much outside experimental error. Fumarate is itself oxidised by guinea-pig liver.

The mechanisms of the oxidations.

It has frequently been suggested that the mechanisms of oxidation of butyric, crotonic and β-hydroxybutyric acids to acetoacetic acid in the liver are related. Friedmann [1908] suggested that β-hydroxybutyric acid occupied an intermediary place between crotonic and acetoacetic acids, and Hurtley [1916] proposed the scheme:

Butyric → crotonic → β-hydroxybutyric → acetoacetic acid.

The most elaborate scheme, which Dakin appears to have favoured [1922], allows three different paths for butyric acid:

$$\begin{array}{c} \text{CH}_3.\text{CH}_2.\text{CH}_2.\text{COOH} \\ \downarrow \qquad\qquad \downarrow \qquad\qquad \downarrow \\ \text{CH}_3.\text{CH}{=}\text{CH}.\text{COOH} \rightleftharpoons \text{CH}_3.\text{CHOH}.\text{CH}_2.\text{COOH} \rightleftharpoons \text{CH}_3.\text{CO}.\text{CH}_2.\text{COOH}. \end{array}$$

The arguments used in favour of these various mechanisms are mainly chemical analogies of more or less value, and none of them is decisive.

Our experiments with inhibitors (propionate and cinnamate, Table VIII) show that the oxidation of β-hydroxybutyric acid is hardly affected by conditions which inhibit considerably the oxidation of butyric and crotonic acids to acetoacetic acid. We can conclude with certainty that neither butyric nor crotonic acid is an intermediary in the oxidation of β-hydroxybutyric acid to acetoacetic acid by guinea-pig liver.

The discovery by Quastel and Wheatley [1934] that ascorbic acid has little effect on acetoacetic acid production by rat liver from β-hydroxybutyric acid, whilst it accelerates acetoacetic acid production from butyric and crotonic acids, provides some evidence that the same statement is true for rat liver.

Let us next consider the view, advocated by several authors, that β-hydroxybutyric acid is an intermediary in the oxidation of butyric and crotonic acids to acetoacetic acid. The only clear evidence we have found in the literature bearing

on this view tells against it, this being the evidence due to Marriott [1914], who stated that when butyrate was injected into a fasting dog a rise in the acetoacetic acid content of the blood preceded a rise in the β-hydroxybutyric acid content.

Our own experiments are also very definitely against the view that β-hydroxybutyric acid is an intermediary. In the first place, we have found some additivity between the acetoacetic acid production from butyric acid and β-hydroxybutyric acid (Table VII). In the second place, we find that the rate of acetoacetic acid production from β-hydroxybutyric acid is definitely lower than from crotonic or butyric acid (Fig. 1 and Table III). An examination of the curves and figures provides quite decisive evidence that β-hydroxybutyric acid cannot be an obligate intermediary between crotonic or butyric acid on the one hand and acetoacetic acid on the other. It remains possible that a part of the crotonic or butyric acid passes through β-hydroxybutyric acid as an intermediary, but there is little need to complicate our views with this possibility, for which there is no positive evidence.

Another view which has been advanced is that crotonic and β-hydroxybutyric acids are interconvertible directly by addition or loss of water. We are aware of no evidence which is inconsistent with the alternative view that in the liver crotonic acid is first oxidised to acetoacetic acid and then reduced to β-hydroxybutyric acid. We prefer the second view, which accounts for the fact that the conversion of crotonic acid into β-hydroxybutyric acid by minced dog liver [Friedmann and Maase, 1913] requires the presence of oxygen. Our conclusions up to the present may be summarised by the scheme:

$$\left.\begin{array}{l}\text{Crotonic acid}\\ \text{Butyric acid}\end{array}\right\rangle \text{acetoacetic acid} \rightleftharpoons \beta\text{-hydroxybutyric acid}$$

We have not yet considered the evidence that acetoacetic acid is convertible into β-hydroxybutyric acid by the liver but may state that adequate evidence is available.

We turn next to a more difficult problem, the relation between the mechanisms of oxidation of butyric and crotonic acids to acetoacetic acid. Our experiments have shown numerous similarities between the rates of the processes and make it fairly certain that a relation exists. For instance, a close numerical relation is found between the rates in different livers (Table VI), which would hardly be likely to exist if the processes were entirely distinct. The reactions are also competitive—when both acids are present the rate is never higher than when one is present alone (Table VII), which is an indication that the modes of oxidation have some feature in common. We will consider the problem first on the usual lines of classical kinetics, according to which if a relation exists, it must, we consider, take one of the three forms:

1. Butyrate → crotonate → acetoacetate.
2. Crotonate → butyrate → acetoacetate.
3. $\left.\begin{array}{l}\text{Crotonate}\\ \text{Butyrate}\end{array}\right\rangle \text{X} \rightarrow \text{acetoacetate}$

The weight of the evidence is against the first possibility. Under all circumstances the rate of formation of acetoacetic acid from butyric acid is greater than that from crotonic acid when both are present at their optimum concentrations. This is shown by numerous figures already given, *e.g.* in Table VI. The effects of inhibitors (Table VIII) on the oxidation of crotonic acid tend to be greater than

on that of butyric acid, which cannot be explained on the first scheme. Nor would it be anticipated that a higher concentration of crotonic acid than of butyric acid would be required to attain the optimum rate.

The second scheme, namely

crotonate → butyrate → acetoacetate

is much more plausible, for the rate of oxidation of butyrate to acetoacetate is always faster than that of crotonate.

One set of facts causes apparent difficulty. Reference to Table II shows that at p_H 7·7–8·0 the rate of oxidation of crotonic acid rises above the level at p_H 7·4, whilst the rate for butyric acid falls below the level at p_H 7·4. Setting the values for p_H 7·4 at 100 for butyric acid and 90 for crotonic acid, values true for a glycerophosphate medium (Table III), it appears that at p_H 7·7–8·0 the rate of oxidation of crotonic acid should be greater than that of butyric acid. But direct comparison of the rates of oxidation of the two acids to acetoacetic acid, at p_H 7·9 in Ringer's solution, shows that even under these conditions butyric acid is oxidised the more rapidly.

Crotonate, however, exerts a secondary inhibitory action on its own oxidation and also on that of butyrate (Table VII), the rate of oxidation of which is lowered to that of crotonate. Taking into account this inhibitory effect, we consider it probable that crotonate is oxidised as fast as butyrate, when both are present at optimum concentrations. We must suppose, therefore, that crotonate is reduced quickly enough to saturate the butyrate-oxidising enzyme, a supposition for which there is no evidence.

It might have been expected that the reduction of crotonate to butyrate would be inhibited by substances that do not affect the oxidation of butyrate. We have, however, found no very striking evidence in favour of greater inhibition of crotonate oxidation than of butyrate oxidation, although certain evidence in this direction exists.

We conclude that this mechanism may be correct, but the evidence is insufficient to establish it.

We turn to the third scheme, which represents the possibility that crotonate and butyrate are oxidised to acetoacetate through a common intermediary. It has already been shown that this cannot be β-hydroxybutyrate, and no other substance suggests itself as a likely intermediary.

In these three mechanisms which have just been considered, the intermediary substances have been considered to be formed as ordinary chemical individuals. A fourth mechanism can be proposed, according to which both crotonate and butyrate are adsorbed by the same enzyme, and both are transformed to acetoacetate while remaining adsorbed on the enzyme surface. In such a process the intermediary stages will not exist as separate molecules, but only in combination with the enzyme. It may be suggested that the reactions which take place are as follows:

$$CH_3.CH_2.CH_2.COO' \rightarrow CH_3.\overset{\times}{C}.CH_2.COO' + 2H$$

$$CH_3.CH{=}CH.COO' \rightarrow CH_3.\overset{\times}{C}.CH_2.COO'$$

$$CH_3.\overset{\times}{C}.CH_2.COO' \xrightarrow[\text{or H acceptor}]{\text{active O}} CH_3.CO.CH_2.COO'$$

A similar method of representing the activation of butyric acid has already been put forward by Quastel [1926]. According to this view, butyrate is desaturated to a product identical with an activated crotonate molecule. The theory is

able to explain the same facts as the second scheme, and also accounts for our failure to find substances which inhibit the oxidation of crotonate to any definite extent without also inhibiting oxidation of butyrate. On the basis of the theory, the facts indicate that crotonate has a lower affinity for the enzyme than has butyrate. We should account most readily for the approximate equality of rates of oxidation of the two acids by assuming that the second stage in the process, which is identical for the two acids, is a slower reaction than the first stage. The evidence is insufficient to decide between the second and fourth schemes proposed. We, however, favour the latter, which in its suggestion of a process taking place entirely on the surface of one enzyme is in line with certain facts related to the oxidation of higher fatty acids, which are discussed in the following paper.

Summary.

1. The rates of oxidation of butyric, crotonic and *dl*-β-hydroxybutyric acids to acetoacetic acid by slices of guinea-pig liver in the presence of oxygen have been investigated.

2. The rate of acetoacetic acid production (Q_{Ac}) varies in the normal manner as a function of substrate concentration with butyric and β-hydroxybutyric acids, but passes through a maximum value when crotonic acid is the substrate.

3. The rate of acetoacetic acid production from butyric and crotonic acids varies with the potassium and calcium ion concentrations in the medium and passes through a maximum value.

4. The optimum p_H for the oxidation is close to the physiological value for butyric and β-hydroxybutyric acids but lies a little to the alkaline side (7·7–7·9) for crotonic acid.

5. Q_{Ac} in presence of butyrate is slightly greater than in presence of crotonate under optimum conditions. The ratio of the rates is fairly constant when measured under definite conditions. Q_{Ac} in presence of β-hydroxybutyrate is considerably lower.

6. There is a definite correlation between the increase in respiration and the increase in Q_{Ac} brought about by the acids.

7. Experiments with mixtures of the acids show that there is competition between butyric and crotonic acids for oxidation, but partial additivity with β-hydroxybutyric acid.

8. Benzoate, cinnamate and phenylpropionate inhibit strongly the oxidation of butyric and crotonic acids to acetoacetic acid. At low concentrations (0·001 M) they appear to inhibit specifically the oxidation of fatty acids.

9. Cinnamate and propionate inhibit the oxidation of β-hydroxybutyric acid to a much smaller extent than the oxidations of butyric and crotonic acids.

10. The evidence supports the scheme of reaction:

$$\left.\begin{array}{l}\text{Crotonate}\\ \text{Butyrate}\end{array}\right\} \text{acetoacetate} \rightleftharpoons \beta\text{-hydroxybutyrate}$$

11. It is possible that crotonate passes through butyrate as an intermediary. It is rather more probable that the process of oxidation to acetoacetate takes place at one and the same enzyme, which effects the complete process with both butyrate and crotonate.

One of the authors (M. J.) wishes to express his thanks to the Medical Research Council for a personal grant. The authors also wish to thank this body for a grant in aid of equipment.

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Biochem. J. 29: 2159-2180, 1935.

CCLVI. STUDIES IN FAT METABOLISM.

II. THE OXIDATION OF NORMAL SATURATED FATTY ACIDS IN THE PRESENCE OF LIVER SLICES.

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(*Received July 11th, 1935.*)

The methods described in Part I of this series [Jowett and Quastel, 1935, 1], together with others described here, have been used to study the oxidation of the normal saturated fatty acids, containing from two to ten carbon atoms, by slices of rat and guinea-pig liver. The work has special reference to the formation of "ketone bodies" as products of oxidation and provides new and quantitative findings.

The results form a contribution to the problem of the mechanism of oxidation of fatty acids. It has been possible to estimate the approximate yields of ketone bodies from some of the fatty acids, which prove to be much higher than those hitherto obtained. The production of ketone bodies is definitely established as a major process in the oxidation; lack of evidence had hitherto led such authorities as Leathes and Raper [1925] to a sceptical view of their importance.

It has also been found that the simple β-oxidation theory of Knoop as applied by Embden to the breakdown of fatty acids in the liver, is unable to account for the facts observed. It will be remembered that according to the view of Embden and Marx [1908] decanoic acid is broken down successively to octanoic, hexanoic and butyric acids before acetoacetic acid is formed.

Experimental methods.

Details of most of the methods used will be found in Part I. Briefly, liver slices have been shaken for two hours at 37° in various media in manometric vessels of the Warburg type. After the tissue slices have been removed to be dried and weighed, the residual solutions are employed for estimation of acetoacetic acid, or for the gravimetric estimation of ketone bodies, described later.

Estimations of acid-base changes have been made in a manner generally similar to that of Negelein [1925]. In these experiments lead perchlorate solution of density about 2·09, prepared according to Krebs [1930], has been used as manometer fluid in order to measure greater outputs of carbon dioxide than can be measured with Brodie's fluid. Lead perchlorate has also been used in a number of other experiments, and it has been found that it leads to an accuracy no less than with Brodie's fluid, since although the manometer changes are halved the readings are steadier.

In estimating the changes in fixed acid, account must be taken of the fact that when the solutions are acidified to decompose bicarbonate the acetoacetic acid present will be partially decomposed. The CO_2 evolution ascribed to bicarbonate will be slightly increased by this decomposition and the subsequent

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CO_2 output in presence of aniline, ascribed to acetoacetic acid, will be lowered. A correction has been calculated on the basis of our measurement of the rate of decomposition of acetoacetic acid in solutions of p_H below 2 (Part I). A correction of 7·5 % is added to the "observed" values for acetoacetic acid, when the solution has been maintained at the low p_H for 15 min. at 37° (for 20 min., 10 % is added). A correction of the same absolute (not relative) amount is added algebraically to the value derived for production of fixed acid. The corrections do not essentially alter the values. The amount of acid added, usually about 0·15–0·2 ml. of N H_2SO_4, has always been sufficient to bring the p_H below 2 (c_H approximately 0·03), allowance being made for the amount of bicarbonate to be decomposed and for the buffering action of salts of weak acids present. The evolution of CO_2 is complete in 10–15 min. After reading the pressure changes the tissue slices are removed, and if acetoacetic acid is to be estimated the solution is brought at once to a p_H near 4·5 by addition of sodium acetate solution, the amount required being usually determined by calculation.

Gravimetric estimation of ketone bodies. The method of Van Slyke [1917] and of Peters and Van Slyke [1932] for blood has been adapted to a micro-scale. All details of the method of precipitation of the mercury compounds have been retained, such as times of refluxing and concentrations of reagents, but the size of apparatus has been reduced, and the volumes of solutions reduced from 175 ml. to 35 or 22 ml. The precipitates are washed on the centrifuge and transferred by suction to Jena sintered glass filters of type G4, which are weighed on a micro-balance.

In the accompanying table are summarised the results of control estimations. Sodium acetoacetate, freshly freed from acetone, has been standardised by the manometric technique of Part I, and the specimen of sodium β-hydroxybutyrate

Control estimations.

Acetoacetic acid.

mg. taken calculated as acetone	Volume during refluxing (ml.)	mg. ppt.	Wt. ppt. / Wt. acetone
0·143	22	2·15	15·1
0·286	,,	5·01	17·5
0·428	,,	8·53	19·9
0·571	,,	11·24	19·7
0·386	35	6·52	16·9
0·445	,,	8·18	18·4
0·857	,,	17·68	20·6

β-Hydroxybutyric acid.

mg. taken calculated as acid	Volume (ml.)	mg. ppt.	Wt. ppt. / Wt. acid
0·280	35	2·69	9·6
0·560	,,	5·36	9·6
0·776	,,	7·52	9·7
0·839	,,	8·75	10·4
1·119	,,	11·09	9·9
1·552	,,	16·38	10·55

used, when dried at 100°, has been taken as pure. For acetoacetic acid the factor tends to be lower for small quantities than Van Slyke's factor of 20, and an empirical correction has been applied for the deviations, although the use of data based on precipitates weighing less than 5 mg. has been almost completely avoided. For β-hydroxybutyric acid the factor 9·85 has been found and employed, which compares with Van Slyke's factor 8·45, and probably indicates a more complete oxidation to acetone than was found by Van Slyke.

Estimations show that in our experiments the quantities of acetoacetic acid and β-hydroxybutyric acid formed from fatty acids are usually of the same order, and to calculate "total ketones" we have therefore assumed that the amounts formed are equal.

The data and assumptions above lead to the following factors by which the weights of precipitates (mg.) are divided in order to convert weights of precipitate into μl. of gas at N.T.P.: acetone + acetoacetic acid 0·052; β-hydroxybutyric acid 0·040, total ketones 0·049.

In our experiments, summarised later, in which ketone bodies are estimated by this method, the contents of the manometer vessel, after removal of the tissue slices, are made up to known volume (usually 10–18 ml.) with water and 1/10 volume of the acid mercuric sulphate reagent of Van Slyke. After filtration through paper, a known volume of filtrate is at once taken for the estimation. Where manometric estimations are also made, these are effected on the contents of another manometer vessel which has undergone simultaneous shaking in the bath with similar solutions and a roughly equal quantity of liver slices.

Manometric method for acetoacetic acid. In applying the manometric method for acetoacetic acid, described in Part I, to the determination of a product derived from higher fatty acids, account must be taken of the possibility that the higher fatty acids may yield homologues of acetoacetic acid, which will probably react with aniline in a similar manner to acetoacetic acid.

Dakin [1923] has shown by liver perfusion experiments that hexanoic acid does not give rise to butyrylacetic acid, but only to acetoacetic acid. He compared the quantities of ketone bodies obtained after distilling the products of oxidation, (*a*) by the iodine titration method, which estimates propyl methyl ketone as well as acetone, and (*b*) by the gravimetric mercuric sulphate method, which estimates only acetone, as was shown by Dakin himself. The question raised is therefore answered for hexanoic acid.

We have found that in our experiments the products obtained react with aniline at the same rate as does acetoacetic acid within the experimental error of 5%. This statement applies to the products of oxidation of hexanoic, octanoic and decanoic acids, and to the product given by rat liver without added fatty acids. In a slightly less quantitative way the same statement may be made with respect to the other fatty acids used, which give small outputs of gas. Now it is probable that higher homologues of acetoacetic acid would react with aniline at rates different from that of acetoacetic acid. This result therefore provides some evidence that acetoacetic acid is the chief β-keto-acid produced in the oxidation of the fatty acids.

Much stronger evidence is derived from a direct comparison of (*a*) the acetoacetic acid as determined by the manometric method, and (*b*) the acetoacetic acid + acetone as determined by the gravimetric methods. Figures of this kind are given later and provide convincing evidence that acetoacetic acid is the only β-keto-acid produced in significant amounts from the fatty acids.

Acetoacetic acid production and respiration of guinea-pig liver in presence of fatty acids.

In Table I are summarised illustrative experiments which show the rates of respiration and acetoacetic acid formation by guinea-pig liver in presence of the normal straight-chain fatty acids containing from two to ten carbon atoms. Every acid raises the respiration, and most of them increase the acetoacetic acid production. Usually there is an optimum concentration for acetoacetic acid production.

Table I. *Acetoacetic acid production and respiration in presence of fatty acids.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Exp.	Fatty acid						
1	Acetic	Conc. (M)	0	0·005	0·01	0·02	
		Q_{O_2}	5·51	7·35	7·00	7·03	
		Q_{Ac}	0·24	0·49	0·49	0·60	
2	Propionic	Conc. (M)	0	0·005	0·01	0·02	
		Q_{O_2}	4·73	8·87	9·68	10·34	
		Q_{Ac}	0·15	0·09	0·12	0·20	
3	Butyric	Conc. (M)	0·0025	0·005	0·01	0·02	
		Q_{O_2}	8·34	9·22	10·1	10·7	
		Q_{Ac}	0·81	1·68	2·49	2·60	
4	Valeric	Conc. (M)	0	0·005	0·01	0·02	
		Q_{O_2}	5·04	10·76	11·44	13·14	
		Q_{Ac}	0·19	0·55	0·61	0·41	
5	Hexanoic	Conc. (M)	0	0·005	0·0075	0·01	
		Q_{O_2}	5·52	11·67	12·37	12·20	
		Q_{Ac}	0·28	3·26	3·40	3·00	
6	Heptanoic	Conc. (M)	0	0·004	0·007	0·01	
		Q_{O_2}	5·03	12·35	13·49	13·90	
		Q_{Ac}	0·28	0·83	0·91	0·84	
7	Octanoic	Conc. (M)	0	0·003	0·006	0·009	
		Q_{O_2}	5·05	7·97	9·39	9·23	
		Q_{Ac}	0·26	1·22	1·44	1·23	
8	Nonanoic	Conc. (M)	0	0·002	0·004	0·006	
		Q_{O_2}	5·84	10·09	10·71	11·28	
		Q_{Ac}	0·34	0·72	0·76	0·78	
9	Decanoic	Conc. (M)	0	0·0015	0·002	0·0025	0·003*
		Q_{O_2}	4·00	6·19	6·78	7·12	7·05
		Q_{Ac}	0·32	1·14	1·12	1·13	1·03

* At this concentration the solubility limit of calcium decanoate in the medium is reached.

Table II summarises the data obtained for the rate of acetoacetic acid production at concentrations of each fatty acid near the optimum. The acids with 4, 6, 8 and 10 carbon atoms produce acetoacetic acid the most rapidly, the 8- and 10-carbon acids being less active than the others. Acetic acid also produces acetoacetic acid, the rate being low but definitely above the very low rate found in its absence. Propionic acid does not produce any acetoacetic acid. Thus far the results are in accord with the findings of Embden and Marx [1908] on dog liver by the perfusion method, and of other workers.

A new result is seen on turning to valeric, heptanoic and nonanoic acids. These acids definitely produce acetoacetic acid, although at rates very much smaller than those characteristic of the neighbouring even-numbered acids. It was considered possible that, although acids of good quality were employed, the preparations of the odd-numbered acids might contain small amounts of the even-numbered acids, which might be responsible for the acetoacetic acid formation. We therefore compared, in one experiment, the rates of acetoacetic acid production using specimens of heptanoic acid obtained from three different sources (an old Kahlbaum specimen; a fresh B.D.H. specimen, and a fresh specimen from Cohen (Berlin), this last being freshly redistilled before use). All three specimens, used at a concentration 0·007 M, gave the same values of Q_{Ac} within the experimental error, the values observed being 1·36, 1·32 and 1·30, whilst the values for the respiration were all very similar. It is very improbable that values of this magnitude could be due to impurities, and that impurities of presumably varying amounts would bring about an equal rate. We therefore conclude that

Table II. *Acetoacetic acid production from fatty acids. Mean values.*

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. of acid (*M*)	No. of exps.	Q_{Ac} (Range)	Q_{Ac} (Mean)	Av. deviation from mean
—	—	42	0·00–0·68	0·20	±0·07
Acetic	0·01–0·02	3	0·23–0·60	0·42	±0·10
Propionic	0·01–0·02	3	0·05–0·20	0·12	±0·05
Butyric	0·01	39	1·09–4·44	2·55	±0·46
Valeric	0·01	2	0·40–0·61	0·51	—
Hexanoic	0·004–0·0075	6	2·19–3·26	2·62	±0·34
Heptanoic	0·004–0·007	4	0·31–1·33	0·88	—
Octanoic	0·005–0·006	5	1·44–2·25	1·80	±0·28
Nonanoic	0·004–0·006	3	0·48–0·92	0·72	—
Decanoic	0·002	3	1·12–1·59	1·39	—

Acid	Approx. optimum concentration for acetoacetic acid production *M*	Approx. optimum concentration for respiration *M*
Acetic	0·01 –	0·01 (?)
Butyric	0·01 –	>0·02
Valeric	0·01	>0·02
Hexanoic	0·007	>0·04
Heptanoic	0·007	>0·01
Octanoic	0·006	0·008
Nonanoic	0·006	>0·01
Decanoic	0·002	0·0025

acetoacetic acid production from fatty acids with odd numbers of carbon atoms is a real phenomenon, which must be taken into account in any theory dealing with the mechanism of oxidation of fatty acids.

No experiments have been made with acids containing more than 10 carbon atoms. With decanoic acid the low solubility of the calcium salt and the lowered rate of acetoacetic acid production were already beginning to make quantitative work difficult, and these difficulties will presumably be greater, though not insurmountable, with the higher acids.

The relation between respiration and acetoacetic acid production.

The data obtained show that there is a real relation between the increase in acetoacetic acid production and the increase in respiration brought about by fatty acids. Table III shows that, at approximately the optimum concentrations

Table III. *Acetoacetic acid oxygen quotients*, i.e. $\frac{Extra\ Q_{Ac}}{Extra\ Q_{O_2}}$

Guinea-pig liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. (*M*)	No. of exps.	Range of quotients	Mean quotient	Mean values of extra Q_{O_2}
Acetic	0·01	3	0·03– 0·17	0·11	1·4
Acetic	0·02	2	0·14– 0·23	0·18	1·5
Propionic	0·01– 0·02	3	−0·02–+0·01	0·00	5·6
Butyric	0·01	7	0·56– 0·69	0·63	3·8
Valeric	0·01	2	0·04– 0·07	0·05	5·7
Hexanoic	0·004–0·0075	4	0·42– 0·55	0·49	4·9
Heptanoic	0·004–0·007	4	0·03– 0·14	0·08	7·9
Octanoic	0·005–0·006	4	0·27– 0·41	0·34	4·3
Nonanoic	0·005–0·006	3	0·08– 0·10	0·09	5·1
Decanoic	0·002	3	0·28– 0·44	0·37	2·8

for acetoacetic acid production, there tends to be a characteristic ratio for each fatty acid. The value of the ratio falls off as the concentration of the fatty acid rises, as can be calculated from many of the data given in Tables I and IV.

This is connected with the fact that whilst acetoacetic acid production passes through a maximum at a certain fatty acid concentration, when the acid has from 5 to 10 carbon atoms, the respiration continues to rise as the concentration is further increased. This in turn may, however, pass through a maximum at a higher fatty acid concentration (Table I, see also Quastel and Wheatley [1933]). The maximum concentrations tend to decrease as we ascend the series of fatty acids.

Table IV. *Inhibitory effect of higher concentrations of fatty acids on acetoacetic acid formation.*

Exp.	Acid		Guinea-pig liver.			
1	Hexanoic	Conc. (M)	0·005	0·01	0·02	0·04
		Q_{O_2}	10·18	11·74	11·82	12·25
		Q_{Ac}	2·63	2·75	2·22	2·0
2	Decanoic	Conc. (M)	0	0·002	0·004*	0·006*
		Q_{O_2}	4·97	7·81	7·39	5·37
		Q_{Ac}	0·31	1·45	1·20	0·82

* In these concentrations calcium decanoate is precipitated in the medium. There is a rapid decrease of the respiration with time in the strongest concentration.

The ratios given in Table III show very clearly the great difference in behaviour between the acids with even numbers of carbon atoms and those with odd numbers. The difference is mainly due to the difference in acetoacetic acid production, but is exaggerated a little by the interesting fact (Table III) that the odd-numbered acids raise the respiration of liver more than do their even-numbered neighbours. This fact suggests that odd-numbered acids are oxidised more completely in the liver than are even-numbered acids.

It is seen in Table IV that raising the concentration of hexanoic acid well above its optimum value depresses the acetoacetic acid formation definitely, but that the inhibition is only partial. A similar effect was observed earlier with crotonic acid. The phenomenon, which is probably fairly general, is presumably an inhibition of a secondary type due to the high adsorption of the fatty acid anions. With decanoate, the inhibitory effect of higher concentrations may be due in part to the precipitation of calcium ion.

Acetoacetic acid production and respiration of rat liver in presence of fatty acids.

The behaviour of rat liver is very similar to that of guinea-pig liver with respect to acetoacetic acid production, but there are a number of differences. Rat liver produces acetoacetic acid faster than does guinea-pig liver from the even-numbered acids (Table V). Butyric, hexanoic and octanoic acids yield acetoacetic acid at very similar rates, whilst the rate with decanoic acid is rather lower.

Rat liver without any added fatty acid yields acetoacetic acid at a definitely measurable rate, several times higher than that with guinea-pig liver. This phenomenon complicates the decision as to whether the other added acids can yield acetoacetic acid. In the case of acetic acid it is clear that acetoacetic acid is produced, and at a rate higher than with guinea-pig liver. Valeric, heptanoic and nonanoic acids do not however affect definitely the nett rate of acetoacetic

Table V. *Mean rates of acetoacetic acid production.*

Rat liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. (*M*)	No. of exps.	Range of Q_{Ac}	Mean Q_{Ac}	Av. deviation from mean
—	—	22	0·74–1·59	1·12	±0·17
Acetic	0·01	4	2·11–2·71	2·38	±0·20
Butyric	0·01	9	3·42–5·68	4·53	±0·57
Valeric	0·01	1	—	1·04	—
Hexanoic	0·007	2	4·59–4·90	4·75	—
Heptanoic	0·007	1	—	1·33	—
Octanoic	0·006	3	4·24–4·68	4·40	—
Nonanoic	0·006	1	—	1·06	—
Decanoic	0·0025	3	3·02–3·99	3·36	—

acid production at the one set of concentrations at which they have been examined (Table VI).

That the odd-numbered acids are oxidised by rat liver is clear from the fact that they increase the respiration. The fatty acids generally do not however raise the respiration of rat liver as much as they do that of guinea-pig liver (Table VII), although they cause greater acetoacetic acid production. A possible explanation is that with rat liver there may be greater competition between the added fatty acids and the substrates already present. However this may be, the fact finds its expression (Table VII) in higher values for the ratio extra acetoacetic acid production/extra respiration for the even-numbered acids.

Table VI. *Acetoacetic acid production and respiration in presence of fatty acids.*

Rat liver. Oxygen. Glycerophosphate buffer.

Exp.	Fatty acid					
1	Acetic	Conc. (*M*)	0	0·01		
		Q_{O_2}	10·27	11·60		
		Q_{Ac}	1·10	2·46		
2	Butyric	Conc. (*M*)	0	0·01		
		Q_{O_2}	9·77	12·74		
		Q_{Ac}	0·96	4·55		
3		Fatty acid	—	Valerate	Heptanoate	Nonanoate
		Conc. (*M*)	—	0·01	0·007	0·006
		Q_{O_2}	11·14	13·24	13·78	15·65
		Q_{Ac}	1·09	1·04	1·33	1·06

Table VII. *Acetoacetic acid oxygen quotients.*

Rat liver. Oxygen. Glycerophosphate buffer.

Acid	Conc. (*M*)	No. of exps.	Range of quotients	Mean quotient	Av. deviation	Extra Q_{O_2}
Acetic	0·01	4	0·45–1·04	0·83	—	1·6
Butyric	0·01	8	0·91–1·33	1·08	±0·12	3·2
Valeric	0·01	1	—	−0·02	—	2·1
Heptanoic	0·007	1	—	0·09	—	2·6
Octanoic	0·006	2	0·65–0·69	0·67	—	5·0
Nonanoic	0·006	1	—	−0·01	—	4·5
Decanoic	0·0025	1	—	0·85	—	2·3

Q_{Ac} *in presence of a mixture of fatty acids.*

In Table VIII an experiment shows that when butyrate and hexanoate are present together, the acetoacetic acid production is not raised above the level shown by either acid separately and is actually no higher than that given by

Table VIII. *Experiments with two added substrates.*

Oxygen. Glycerophosphate buffer.

Exp. 1. Guinea-pig liver	Conc. butyrate (M)	—	0·02	—	0·02
	Conc. hexanoate (M)	—	—	0·006	0·006
	Q_{O_2}	4·21	9·72	8·41	10·42
	Q_{Ac}	0·14	3·10	2·32	2·18
Exp. 2. Rat liver	Conc. butyrate (M)	—	0·01	0·01	
	Conc. acetate (M)	—	—	0·01	
	Q_{O_2}	9·43	12·34	13·05	
	Q_{Ac}	1·00	4·13	5·00	

the acid showing the lower rate of production. A similar result was previously obtained for crotonic and butyric acids. Competition of this kind is probably a general phenomenon, indicating some common mode of oxidation for the different fatty acids.

Acetic acid however proves to be an exception (Table VIII), for Q_{Ac} in presence of butyrate and acetate is higher than when the acids are present alone. It would be expected that the mechanism of formation of acetoacetic acid from acetic acid would be of quite a different type from that involved in the oxidation of other fatty acids, and this result therefore accords with expectation.

The formation of β-hydroxybutyric acid and acetone.

It may appear to have been tacitly assumed in previous discussions, particularly in Part I, that acetoacetic acid is the only product of fatty acid oxidation which concerns us. This of course is not true, for acetoacetic acid is transformed in the liver in two ways.

One possible mode of breakdown of acetoacetic acid in liver is to acetone and carbon dioxide. Some evidence that acetone as well as acetoacetic acid resulted from fatty acid oxidation in the perfused dog liver was obtained by Embden and Engel [1908], but the decomposition may have been due to the perfusing blood, since according to Grégoire [1933] dog blood decomposes acetoacetic acid to acetone. A few figures we have obtained (Table IX) suggest that some acetone is formed during oxidation of fatty acids by rat liver. The rate of formation of acetoacetic acid + acetone, estimated gravimetrically, is greater than the rate of acetoacetic acid formation estimated manometrically, and probably therefore acetone is being formed from acetoacetic acid. A more definite and interesting conclusion to be drawn from the figures is that acetoacetic acid is the only β-keto-acid formed to an appreciable extent in the experiments. If a higher homologue of acetoacetic acid were formed it would probably be estimated by the manometric method but not, in view of Dakin's experiments already mentioned, by the gravimetric method.

Acetoacetic acid is also transformed in another manner in liver, namely to β-hydroxybutyric acid. We have already shown that in the oxidation of butyric and crotonic acids by guinea-pig liver β-hydroxybutyric acid is not an intermediary product in the formation of acetoacetic acid. In these cases therefore the formation of β-hydroxybutyric acid results from a reduction of acetoacetic acid, and it is likely that the same is true for other fatty acids. The figures given in Table IX show that quantitatively β-hydroxybutyric acid is an important product of fatty acid oxidation in the liver, the amount formed in most of our experiments being of the same order as that of acetoacetic acid. Formation of β-hydroxybutyric acid by the liver has been shown to take place from butyric acid [Friedmann and Maase, 1910, and others] and from hexanoic acid [Dakin,

Table IX. *Gravimetric estimations of ketone body formation.*

The symbol Q_{Ac}, hitherto used to indicate rate of acetoacetic acid production by the manometric method, is also used to indicate rate of formation of acetoacetic acid + acetone, by the gravimetric method, and is then always distinguished by the statement that the gravimetric method was used.

Q_{oxy} indicates similarly the rate of formation of β-hydroxybutyric acid, and Q_{ket} the rate of formation of total ketones, both estimated gravimetrically.

Rat liver.

Medium—Ringer's solution of initial p_H 7·4.
Either 1·5% CO_2, 0·008 *M* $NaHCO_3$; or 5% CO_2, 0·025 *M* $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{Ac} (grav.)	Ratio
1	Butyrate	1·74	1·96	0·89
1	Hexanoate	1·27	1·51	0·84
2	Octanoate	1·52	2·16	0·70
		Q_{Ac} (grav.)	Q_{oxy}	
3	Butyrate	2·89	1·62	
1	Butyrate	1·96	1·40	
2	Octanoate	2·16	2·58	
		Q_{Ac} (manom.)	Q_{ket}	Ratio
1	Butyrate	1·74	3·36	0·52
1	Hexanoate	1·27	2·08	0·61
4	Octanoate	1·62	1·61	0·63
2	Octanoate	1·52	4·74	0·32
4	Decanoate	1·20	2·22	0·54
2	Decanoate	1·27	3·42	0·37

Guinea-pig liver.

Ringer's solution: 1·5% CO_2, 0·009 *M* $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{ket}	Ratio
5	Butyrate	2·71	4·07	0·67
6	Valerate	0·95	2·11*	0·45 (?)
7	Hexanoate	1·50	2·94	0·51
8	Heptanoate	0·80	1·30	0·61
9	Octanoate	0·73	0·79	0·92
10	Octanoate	1·22	1·37	0·89
11	Decanoate	0·81	1·55	0·52

* This value is too high, for, it has been found, in confirmation of the work of Butts *et al.* [1935], that valeric acid itself gives a precipitate in the Van Slyke estimation.

Ringer's solution: 5% CO_2, 0·025 *M* $NaHCO_3$.

Exp.	Substrate	Q_{Ac} (manom.)	Q_{ket}
5	Butyrate	2·71	4·07
5	Butyrate + 0·002 *M* benzoate	1·53	2·25
5	% inhibition	43	45
		Q_{oxy}	
12	Butyrate 0·01 *M*	0·60	
12	Acetoacetate 0·01 *M*	1·18	

1923], but as far as we are aware it has not previously been shown to take place from other normal saturated fatty acids.

A very rough estimate of the average ratios of the substances making up the total ketone bodies produced from fatty acids by rat liver in our experiments is acetoacetic acid 0·5, acetone 0·1, β-hydroxybutyric acid 0·4. The proportions will not necessarily be the same for other experimental conditions. The breakdown of

acetoacetic acid to acetone is almost certainly irreversible, and the proportion of acetone will therefore increase with time. The ratio of acetoacetic acid to β-hydroxybutyric acid varies in our experiments, and the nett rate of transformation of one of these substances into the other will presumably depend on the supply of other metabolites to the liver cells. The cells will tend to set up a dynamic equilibrium between these two substances, the position of the equilibrium being dependent on the conditions.

Accepting, as we do, the view that acetone and β-hydroxybutyric acid are products derived from acetoacetic acid, it is important for us to know whether the nett acetoacetic acid formation in any one liver bears a constant ratio to the formation of total ketone bodies, for on this view the total ketone bodies measure the gross acetoacetic acid formation. It is, for instance, desirable for us to know whether a substance that inhibits the nett acetoacetic acid production to a certain extent inhibits also the gross acetoacetic acid production to an equal extent.

We cannot claim that this is always the case, but where the point has been tested by us it is true. An experiment recorded in Table IX shows that benzoate inhibits the nett formation of acetoacetic acid from butyric acid by guinea-pig liver to the same extent as it inhibits total ketone formation. Similar evidence of a more qualitative kind regarding the inhibiting action of benzoate is presented later.

If β-hydroxybutyric acid is derived from acetoacetic acid, and not from butyric acid more directly, it would be expected that acetoacetic acid might yield β-hydroxybutyric acid at a rate faster than butyric acid does. This expectation has been verified by an experiment with guinea-pig liver (Table IX), which therefore supports the views put forward.

The yield of ketone bodies from fatty acids.

In the experiments recorded in other sections of this work we have endeavoured to maintain the fatty acid concentrations at levels which do not fall very much with time, so that the rates of oxidation are measured under fairly definite conditions.

In this section, on the other hand, we present experiments in which the object has been to allow the tissue to remove the fatty acid completely during the experiment, the period of which has however been restricted as before to two hours. The fatty acid concentrations have been lowered and the quantity of tissue increased. Several manometer vessels have been set up in each experiment. In each vessel the volume of solution and the initial concentration of the fatty acid have been the same, but the weight of tissue has been varied. By comparing the quantities of ketone bodies formed in the presence of varying weights of tissue, it should be possible to tell whether any of the quantities of tissue has been sufficient to transform the fatty acid completely, for when this object is attained the quantity of ketone bodies formed will be expected to become constant. We appear to have been successful in this object, but two difficulties arise in the exact interpretation of the experiments.

(1) In the absence of added fatty acids, liver gives rise to some acetoacetic acid. Increasing the quantity of tissue indefinitely will therefore probably increase the quantity of ketone bodies indefinitely. We can to some extent meet the difficulty (*a*) by choosing a liver with relatively low "spontaneous" acetoacetic acid production, *i.e.* guinea-pig liver rather than rat liver, and (*b*) by attempting to correct for the "spontaneous" acetoacetic acid formation.

(2) A second source of error, which however is small in our experiments, is the necessity of correcting for the quantity of ketone bodies removed from the vessels with the tissue slices. We find that the loss in weight of the vessels when the tissue slices are removed is for guinea-pig liver about six times the dry weight of the slices found subsequently. We have calculated the correction to be applied on the basis that the ketone bodies removed are equal to the quantity contained in an equal quantity of solution. As there may not be equal partition of ketone bodies between liver slices and medium, the assumption may lead to an error of a few per cent. in our results[1].

Details of one of our experiments are recorded in Table X, which make clear the nature and magnitude of the corrections applied. The results of the experiments are shown in Table XI, where the several values obtained in each experiment, both corrected and not corrected for the "spontaneous" acetoacetic acid formation, are shown.

Table X. *Experiment showing yield of acetoacetic acid from hexanoic acid.*

O_2. 3·2 ml. of solution containing glycerophosphate buffer. Guinea-pig liver.

Vessel ...	1	2	3	4
Initial conc. hexanoate (*M*)	—	0·0025	0·0025	0·0025

After 117 min. shaking at 37°, tissue removed from vessels, to which were added 0·2 ml. aniline hydrochloride and 0·3 ml. *N* acetic acid. $v_p = 3·7$.

Dry wt. tissue (mg.)	27·6	48·0	39·7	21·4
t_{cor}	1·393	1·492	1·548	1·553
Δh (aniline)	+18·1	+118·1	+107·2	+ 75·3
µl. CO_2 (uncorrected)	25·2	176·2	165·0	116·9
µl. CO_2 (corrected for aniline blank)	13·2	164·2	153·9	104·9
µl. CO_2 (corrected for loss due to tissue removal)*	13·9	178·9	165·3	109·1
µl. CO_2 (corrected for spontaneous acetoacetic acid formation)†	0	154·7	145·3	98·3
µl. hexanoate added	0	179·2	179·2	179·2
* Acetoacetic / Hexanoic (uncorrected)	—	1·00	0·92	0·61
† Acetoacetic / Hexanoic (corrected)	—	0·86	0·81	0·55
Q_{O_2} (14–117 min.)	4·59	7·82	7·80	8·13
Q_{Ac} (0–117 min.)	0·26	1·91	2·14	2·61

In Table XI are also shown the results of a similar series of experiments carried out in the presence of 0·04 *M* sodium malonate. Malonate raises the spontaneous acetoacetic acid formation in guinea-pig liver [Jowett and Quastel, 1935, 2] and it was thought that by increasing the correction for this it would be made clear whether or not this correction should be applied. It was found later, however, that malonate inhibits the breakdown of acetoacetic acid by liver, and the complete interpretation of the experiments is, therefore, difficult.

The first set of experiments may be taken to show that butyric acid yields about 0·3–0·5 mols. of acetoacetic acid, hexanoic about 0·9, octanoic 0·6, and decanoic acid 0·6.

The highest yields of acetoacetic acid previously obtained from these fatty acids were derived from the experiments of Embden and Marx [1908] with dog

[1] It may be pointed out that the correction discussed has not usually been applied to experiments in other sections of this work. For 20 mg. of guinea-pig liver in 3·2 ml. solution, the correction amounts only to 4%, and for relative results in any one experiment is much less.

Table XI. *Yields of ketone bodies from fatty acids.*

Guinea-pig liver.

Acid	Initial conc. *M*	µl. fatty acid	Acetoacetic acid in mols. per mol. of fatty acids. Several weights of tissue were employed in each experiment, the result for the largest weight being at the left of each column: Uncorrected			Corrected		
Butyric	0·004	285	0·49	0·42	0·36	0·45	0·39	0·33
Butyric	0·004	285	0·55	0·49	0·36	0·38	0·34	0·28
Butyric	0·005	357	0·40	0·40	0·27	0·31	0·33	0·23
Hexanoic	0·0025	179	1·00	0·92	0·61	0·86	0·81	0·55
Octanoic	0·0015	107	0·79	0·84	0·75	0·55	0·64	0·60
Decanoic	0·0015	107	0·84	0·68	0·65	0·48	0·48	0·48
Decanoic	0·002	143	0·92	0·83	0·80	0·64	0·64	0·64
Yields of acetoacetic acid. In presence of malonate.								
Hexanoic	0·0025	179	1·32	1·18	1·07	0·89	0·85	0·82
Octanoic	0·002	143	1·51	1·28	—	0·88	0·92	—
Decanoic	0·002	143	1·27	1·14	—	0·70	0·74	—
Decanoic	0·002	143	1·35	1·19	—	0·63	0·69	—
Yields of total ketones. Mols. per mol. of fatty acid.								
Butyric	0·0029	223	0·36	0·35	0·28	0·22	0·26	0·21
Hexanoic	0·0023	179	1·29	1·26	1·06	1·16	1·15	0·98
Octanoic	0·002	142	1·14	1·27	1·21	0·96	1·11	1·11
Decanoic	0·002	156	0·86	0·85	—	0·66	0·69	—

livers. These figures show a yield of 0·12–0·18 mols. of acetone + acetoacetic acid per mol. of fatty acid, uncorrected for the "spontaneous" production by the liver. It was not proved or even suggested in these experiments that the fatty acids added were completely removed by the liver. The low values of the figures have however made Leathes and Raper [1925] sceptical of the importance of ketone bodies in fatty acid metabolism, and it is only evident now that acetoacetic acid is a quantitatively important product of fatty acid oxidation.

An even more interesting figure than the yield of acetoacetic acid is the yield of total ketones from the fatty acids. A few experiments have been made to determine total ketone yields on similar lines to those carried out with acetoacetic acid, and they are summarised in Table XI. The medium in these experiments was Ringer's solution (5% CO_2, 0·025 *M* $NaHCO_3$). In the "blank" vessels, *i.e.* those to which no fatty acid was added, the quantity of total ketones found as mercury precipitate did not exceed 0·44 mg., which corresponds to a value of Q_{ket} of 0·09.

The Van Slyke method is probably inaccurate for the small precipitates in question, and we have assumed a "blank" value $Q_{ket} = 0·2$ for the purpose of calculating "corrected" values for the yield of total ketones. The experiments give us values of 1·15–1·3 mols. of total ketones from hexanoic acid, 1·1–1·3 from octanoic acid, and 0·7–0·85 from decanoic acid. The value found with butyric acid, 0·2–0·35, is probably rather too low, in view of the higher yield of acetoacetic acid previously found.

We cannot state with certainty that any of the values definitely exceeds one molecule of total ketones per molecule of fatty acid, and the results may be

summarised as follows: hexanoic and octanoic acids each yield about one molecule of ketone bodies, decanoic acid slightly less and butyric acid $\frac{1}{2}$–$\frac{1}{3}$ molecule.

The yield of ketone bodies from butyric acid is much less than that from the higher acids, and it is therefore very unlikely that the higher acids yield ketone bodies exclusively by a mechanism by which they first break down to butyric acid.

It is possible that a proportion of the fatty acid is broken down by a path that does not lead to ketone body formation, and the greater yields of ketone bodies which have been found with fatty acids higher than butyric acid may be explained on the following lines: (*a*) acetic acid may be one of the products of breakdown of higher fatty acids, and this may be an additional source of acetoacetic acid, or (*b*) octanoic and decanoic acids may break down into two units containing four carbon atoms and thereby yield two molecules of acetoacetic acid[1].

It is unlikely, however, that the production of acetic acid from hexanoic acid will explain the very considerable difference between the yields of acetoacetic acid from this acid and from butyric acid, for this would necessitate the complete conversion of acetic acid into acetoacetic acid.

This section may be concluded by giving some values (Table XII) for the acetoacetic acid formed per molecule of fatty acid added, which were obtained in experiments not specially designed for obtaining complete removal of the fatty acid. The figures given are therefore lower than the actual yields that could be obtained, but may serve for the present to indicate minimum values of the yield of acetoacetic acid, for some cases where a better approximation is not yet available. The figures are sufficient to show that guinea-pig liver produces acetoacetic acid from heptanoic and nonanoic acids to a small but definite extent. It is also clear that rat liver produces acetoacetic acid from the even-numbered fatty acids to a considerable extent.

Table XII. *Yields of acetoacetic acid.*

(Not maximum values.)

Liver	Acid	Initial conc. *M*	µl. fatty acid	Yield, mols. acetoacetic acid per mol. fatty acid: Uncorrected	Yield, mols. acetoacetic acid per mol. fatty acid: Corrected for blank
Guinea-pig	Acetic	0·005	357	0·08	0·04
"	Valeric	0·005	357	0·06	0·04
"	Heptanoic	0·007	500	0·18	0·14
"	Nonanoic	0·002	143	0·31	0·16
Rat	Acetic	0·01	714	0·16	0·08
"	Butyric	0·01	714	0·45	0·34
"	Octanoic	0·006	428	0·29	0·23
"	Decanoic	0·0025	178	0·88	0·58

Acid-base changes during fatty acid oxidation.

A series of experiments is summarised in Table XIII in which the change in the bicarbonate concentration of the Ringer's solution employed as medium is used to measure the production or disappearance of fixed acid during the oxidation of fatty acids by guinea-pig liver. The symbol Q_s, defined on similar lines to the others employed, denotes acid production. As is usual, no attempt has

[1] The probability of this latter mode of breakdown is enhanced by the conclusion drawn, in work recently published by Butts *et al.* [1935], that the intact rat can break down octanoic acid to two molecules of ketone bodies. These authors consider that in the rat butyric and hexanoic acids yield about one molecule of ketone bodies.

Table XIII. *Acid production during fatty acid oxidation.*

Guinea-pig liver.
Ringer's solution: $O_2 + 1{\cdot}5\%\ CO_2$, 0·009 *M* $NaHCO_3$.

Exp.	Added substrate	Conc. *M*	Q_S	Q_{Ac}	Q_{Ket}	$\frac{Q_S}{Q_{Ac}}$	$\frac{Q_S}{Q_{Ket}}$
1	—	—	+0·29	0·19	—	—	—
2	—	—	+0·40	0·25	—	—	—
3	—	—	+0·25	0·09	—	—	—
4	—	—	+0·35	—	—	—	—
5	—	—	+0·51	0·14	—	—	—
6	—	—	+0·38	—	—	—	—
5	Acetate	0·005	−0·39	0·38	—	−1·0	—
4	Acetate	0·009	−1·23	—	—	—	—
6	Propionate	0·009	−3·25	—	—	—	—
7	Propionate	0·01	−2·35	—	—	—	—
3	Butyrate	0·006	−0·04	3·06	—	−0·01	—
8	Butyrate	0·01	−0·17	—	—	—	—
9	Valerate	0·01	−1·86	0·95	2·11*	−2·0	−0·9*
2	Hexanoate	0·006	+2·20	2·93	—	+0·8	—
10	Hexanoate	0·0075	+1·40	1·50	2·94	+0·9	+0·5
11	Heptanoate	0·007	+0·52	0·80	1·30	+0·6	+0·4
12	Octanoate	0·004	+2·50	2·22	—	+1·1	—
13	Octanoate	0·004	+1·95	1·75	—	+1·1	—
1	Octanoate	0·004	+1·97	2·03	—	+1·0	—
14	Octanoate	0·006	+0·63	0·73	0·79	+0·9	+0·8
15	Octanoate	0·006	+0·91	1·22	1·37	+0·7	+0·7
16	Decanoate	0·0025	+1·10	0·81	1·55	+1·4	+0·7

* See footnote to Table IX.

been made to correct values of Q_S for the buffering power of the tissue slices, or for the very slight buffering due to the bicarbonate-carbonate system, and the results will all be numerically a little too low.

The results are illuminating, although not very numerous. It may first be noted that there is evidence of a definite parallelism between Q_S and Q_{Ac} in the cases where several experiments have been done with any one acid. The parallelism provides some evidence that the acid-base changes are directly connected with fatty acid oxidation and are not due to a secondary stimulation or inhibition of other processes in the liver.

Hexanoic, octanoic and decanoic acids all show acid production definitely exceeding the small acid production by liver in the absence of added fatty acid. Butyric acid on the other hand shows a slight disappearance of acid as compared with the control.

The reactions

$$CH_3.CH_2.CH_2.COO' \rightarrow CH_3.CO.CH_2.COO' \rightarrow CH_3.CH(OH).CH_2.COO'$$

which occur with butyrate lead to no acid-base change, while the reaction

$$CH_3.CO.CH_2.COO' + H_2O \rightarrow CH_3.CO.CH_3 + HCO_3'$$

which probably occurs to a small extent, leads to disappearance of fixed acid. It may be concluded that any other type of oxidation which butyrate may undergo does not lead to much nett appearance or disappearance of acid groups.

The reactions occurring with the higher even-numbered acids may on two views at least be expected to lead to production of acid.

(1) The reaction with hexanoic acid may be

$$CH_3.CH_2.CH_2.CH_2.CH_2.COO' \rightarrow CH_3.CO.CH_2.COO' + CH_3.COOH,$$

and on the same lines the higher acids would give two or more molecules of acetic acid. It must be made clear that there is no evidence whatever, of which

we are aware, that acetic acid is a product of fatty acid oxidation. That any acid at all is produced during the oxidation of fatty acids by liver, beyond acetoacetic and β-hydroxybutyric acids, is a fact which has not been shown prior to the present work.

The hypothesis that acetic acid is formed is not easy to test from our data. Reactions such as the one formulated lead to quite definite ratios between the acid production that should be observed and the formation of acetoacetic acid + β-hydroxybutyric acid. With hexanoic acid the ratio Q_S/Q_{ket} should be 1, with octanoic 2, with decanoic 3. The position is complicated by the fact that part of the acetic acid hypothetically formed would react further, and in such a way as to lower the expected ratios below the values given.

Acetic acid itself forms acetoacetic acid, and furthermore, if the process is a condensation,

$$2CH_3.COO' \rightarrow CH_3.CO.CH_2.COO' + OH',$$

fixed acid will disappear. Disappearance of fixed acid is observed, in fact, with acetic acid in liver, but its amount is probably greater than can be accounted for by the rate of acetoacetic acid formation, and it may be that some of the acetic acid is oxidised completely, with disappearance of fixed acid.

In spite of the complications, it would be expected on the hypothesis that the ratio Q_S/Q_{ket} would rise as we pass from hexanoic to octanoic and from octanoic to decanoic acid. The observed ratios (0·5, 0·7, 0·7), however, do not lead to a definite conclusion. Attempts to isolate acetic acid as a product of fatty acid oxidation should settle the question. A few experiments we have made in this direction have as yet given no definite results.

(2) It has been found by Verkade and van der Lee [1934, 1] that when human subjects are fed on glycerides made from pure saturated fatty acids, the reaction

$$CH_3.(CH_2)_n.COOH \rightarrow HOOC.(CH_2)_n.COOH$$

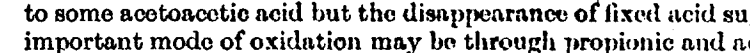
takes place in the body to some extent when $n+2$ has values of 8 to 12, for small quantities of the dibasic acid are excreted in the urine. Such a process as that formulated would lead to the observation of fixed acid production in our experiments. It is, however, unknown whether the production of dibasic acids takes place in the liver or elsewhere in the body. The yields of dibasic acid in the urine are also small in relation to the quantity of fatty acid ingested—octanoic acid giving up to 0·18% and decanoic acid up to 2·2% of the yield theoretically possible—and it is therefore unknown whether a considerable proportion of the fatty acid is oxidised in this way in the human body. From our results on the total ketone production from hexanoic acid, we conclude that hexanoic acid at least does not undergo oxidation in guinea-pig liver to dibasic acid as a final product to any appreciable extent, hence it is unlikely that the acid production observed in this case can be due to the formation of dibasic acids.

The reaction formulated, however, together perhaps with degradation to lower dibasic acids [Verkade and van der Lee, 1934, 2], cannot at present be excluded as a possible partial path of oxidation for a number of the fatty acids.

Let us now consider the problem of the oxidation of the odd-numbered acids. It will be seen that when propionic acid is oxidised, there is a considerable disappearance of fixed acid. This may be due to complete oxidation of some of the propionic acid, and perhaps, as already suggested [Dakin, 1922], lactic and pyruvic acids are intermediaries in the oxidation.

With valeric acid fixed acid also disappears, whilst with heptanoic acid there is apparently slight acid production. With both these acids formation of acetoacetic acid is higher than would be expected if acetic acid is the inter-

mediary that gives rise to it. It is probable that valeric acid gives rise directly to some acetoacetic acid but the disappearance of fixed acid suggests that a more important mode of oxidation may be through propionic and acetic acids or their partially oxidised derivatives. The reaction may be primarily:



$$CH_3.CH_2.CH_2.CH_2.COOH \rightarrow CH_3.CH_2.COOH + CH_3.COOH$$

the propionic acid undergoing complete oxidation, the nett result being a disappearance of fixed acid but rather less than in the case of propionic acid alone owing to the presence of the hypothetical acetic acid.

When heptanoic acid is oxidised we imagine the same process extended, so that a molecule of propionic acid and two molecules of acetic acid are formed. The nett result of this process would be greater fixed acid production than in the case of valeric acid. Acetoacetic acid production from heptanoic acid cannot be explained as dependent upon the formation of acetic acid.

It will have been observed in Table XIII, that where a weakly buffered Ringer's solution was used, the production of acetoacetic acid from valeric and heptanoic acids compared with those from the even-numbered acids seems high relative to the figures previously obtained in a glycerophosphate medium. It occurred to us that this phenomenon might be due to a relatively low p_H in these experiments, caused by the greater acidifying effect of respiratory carbon dioxide when the initial carbon dioxide content of the gas mixture is low (1·5%). We have therefore compared (Table XIV) the relative activities of the odd- and even-numbered acids in more strongly buffered media and have obtained some indication that at a lower p_H the acetoacetic acid production of the odd-numbered acid is raised relatively to that of a neighbouring even-numbered acid.

Table XIV. *Effect of* p_H *on relative acetoacetic acid production from odd- and even-numbered acids.*

Guinea-pig liver. Ringer's solution, 95% O_2 + 5% CO_2.

			Q_{Ac} in presence of				
Exp.	Initial $[HCO_3']$	Approx. p_H	No addition	Valerate 0·01 *M*	Hexanoate 0·0075 *M*	Heptanoate 0·007 *M*	Octanoate 0·006 *M*
1	0·025	7·4	—	0·49	2·25	0·61	1·52
2	0·0125	7·1	0·10	0·84	2·03	0·92	—

The effect of benzoate on the oxidation of fatty acids.

We have already shown in Part I that benzoate at low concentrations inhibits the oxidation of butyric and crotonic acids to acetoacetic acid by guinea-pig liver. The same substance has been examined here with regard to its inhibiting action on the formation of acetoacetic acid from fatty acids by rat liver.

As will be seen from Table XV, 0·002 *M* benzoate lowers Q_{Ac} by some 50% (*a*) when there is no substrate added, (*b*) when acetic acid is present and (*c*) when the substrate is butyrate. It should be noted that the respiration in the absence of added substrates is unaffected by benzoate at concentrations up to 0·01 *M*. Benzoate at low concentrations is evidently quite a specific poison for tissue processes.

Benzoate, however, in contrast to its effect on Q_{Ac} in presence of acetate and butyrate, has a much smaller effect on the production of acetoacetic acid from hexanoic, octanoic and decanoic acids (Table XV).

As a result, when the acetoacetic acid productions in the presence of benzoate from the various fatty acids are compared in any one experiment it is found that Q_{Ac} from butyrate is lower than that from hexanoic, octanoic and decanoic acids.

Table XV. *Effect of benzoate on formation of acetoacetic acid.*

Rat liver. Oxygen. Glycerophosphate buffer.

Substrate (M)	% decrease in Q_{Ac} in presence of 0·002 M benzoate		
—	53	32	46
Acetate 0·01	51	63	56
Butyrate 0·01	63	48	45
Hexanoate 0·007	13		
Octanoate 0·006	18	20	
Decanoate 0·0025	5		

Values of Q_{Ac} in presence of 0·002 M benzoate.

Exp.	Butyrate	Hexanoate	Octanoate	Decanoate
1	2·11	4·01	—	—
2	1·70	—	3·45	—
3	1·81	—	3·44	—
4	2·66	—	—	3·79

Values of Q_{Ac} in presence of benzoate.

Exp.	Concentration benzoate (M)	Butyrate 0·02 M	Hexanoate 0·007 M	Octanoate 0·006 M	Decanoate 0·0025 M
5	0	4·70	4·90	4·68	3·07
6	0·005	2·62	4·22	4·57	3·12
7	0·0075	1·77	3·09	3·97	3·10
8	0·01	2·59	3·92	4·09	4·01

Same as above in relative units. Values of Q_{Ac} in presence of benzoate.

Exp.	Concentration benzoate	Butyrate	Hexanoate	Octanoate	Decanoate
5	0	153	160	152	(100)
6	0·005	84	135	146	(100)
7	0·0075	57	100	128	(100)
8	0·01	65	98	102	(100)

When the concentration of benzoate is increased, it is possible to find conditions where, similarly, Q_{Ac} from hexanoate is lower than that from octanoic acid. Probably conditions of benzoate concentration could be found under which decanoic acid would form acetoacetic acid faster than do octanoic and hexanoic acids.

In the absence of benzoate the values of Q_{Ac} are approximately equal with butyrate, hexanoate and octanoate, but rather lower with decanoate. The behaviour in absence of benzoate may thus be compatible with the β-oxidation mechanism according to which decanoic acid is transformed successively into octanoic, hexanoic and butyric acids before acetoacetic acid is formed. The behaviour in presence of benzoate, however, is very difficult to explain on this theory of fatty acid breakdown.

Before considering the subject further, we should note that it has also been found that the same alteration in relative rates with the different fatty acids by benzoate is shown when we measure the total ketone production instead of acetoacetic acid production. Reference to Table XVI shows in the case of rat liver that the findings are the same, and that the total ketone production from butyric acid can be lowered below the level shown by the other fatty acids.

These results clearly compel us to abandon the mechanism proposed by Embden. The formation of ketone bodies from the higher fatty acids appears not to be necessarily preceded by their degradation to lower fatty acids and

Table XVI. *Total ketone production in presence of benzoate.*

Ringer's solution (95% O_2 + 5% CO_2; 0·025 *M* $NaHCO_3$).

	Q_{Ket} in presence of			
	Butyrate 0·02 *M*	Hexanoate 0·007 *M*	Octanoate 0·006 *M*	Decanoate 0·0025 *M*
Rat liver, 0·01 *M* benzoate	1·54	2·48	3·13	1·95
Rat liver, 0·015 *M* benzoate	2·28	3·08	2·63	—
Guinea-pig liver, 0·01 *M* benzoate	1·39	0·87	—	—
Guinea-pig liver, 0·01 *M* benzoate	0·85	0·54	0·61	0·71

finally to butyric acid before ketone bodies are formed. No escape from this conclusion seems possible. In our experiments butyric acid is the acid with which optimum conditions are most easily obtained, since excessive concentrations do not affect the oxidation to ketone bodies. It is much more certain that conditions are optimum for butyrate than for the higher fatty acids, and yet in presence of benzoate it forms ketone bodies at a lower rate than do the higher fatty acids.

It remains to consider our experiments further in the light of one or two possibilities.

(1) It is possible that a part of the ketone bodies from the higher fatty acids arises through acetic acid which may be split off from them as an intermediary. Our experiments with acetic acid show however that acetoacetic acid production from it is as much inhibited by benzoate as is that from butyrate, and is in any case small. The view that acetic acid is an intermediary is therefore of little importance in this connection.

(2) In the case of octanoic and higher acids, it is conceivable that a molecule of fatty acid might give rise to two or more molecules of acetoacetic acid, and that one of these molecules might be formed through butyric acid as an intermediary. On this view the inhibition of ketone body formation in the case of octanoic acid should be at least half as great as with butyric acid.

In the case of guinea-pig liver the inhibitory effect of benzoate does not vary so much from one fatty acid to another (Tables XVI and XVII), and we cannot conclude from our experiments on this tissue with benzoate that the Embden mechanism is invalid. Apparently fluoride, like benzoate, inhibits acetoacetic acid production from the higher fatty acids rather less than from butyric acid.

Table XVII. *Effect of inhibitors on guinea-pig liver.*

Exp. 1. (Ringer's solution: 5% CO_2; 0·025 *M* $NaHCO_3$.)					
Conc. butyrate (*M*)	0·01	0·01	—	—	
Conc. octanoate (*M*)	—	—	0·01	0·01	
Conc. benzoate (*M*)	—	0·002	—	0·002	
Q_{Ac}	1·78	0·74	1·79	0·90	
% decrease in Q_{Ac}	—	58	—	50	
Exp. 2. (Glycerophosphate buffer.)					
Conc. butyrate (*M*)	—	0·01	0·01	—	—
Conc. octanoate (*M*)	—	—	—	0·006	0·006
Conc. fluoride (*M*)	—	—	0·0031	—	0·0031
Q_{O_2}	4·66	8·18	5·67	9·25	6·12
Q_{Ac}	0·18	2·27	0·67	1·93	0·84
% decrease in Q_{Ac}	—	—	70	—	57
Exp. 3. (Glycerophosphate buffer.)					
Conc. butyrate (*M*)	—	—	0·01	0·01	
Conc. arsenite (*M*)	—	0·001	—	0·001	
Q_{O_2}	4·53	1·96	8·68	2·23	
Q_{Ac}	0·20	0·02	2·05	0·40	

An experiment in Table XVII shows in an interesting way how much more specific fluoride and benzoate are in their actions on acetoacetic acid production than is arsenite. Arsenite inhibits acetoacetic acid production strongly, but the respiration also is greatly inhibited, showing that other tissue-processes are greatly affected.

THE MECHANISMS OF THE OXIDATIONS.

The β-oxidation theory of Knoop proposes that fatty acids are oxidised in a series of stages, in each of which the β-carbon atom of a fatty acid is attacked in such a manner that loss of two carbon atoms follows. The experiments of Embden led him to suggest that for the even-numbered fatty acids the stages led through the following substances:

decanoic acid → octanoic acid → hexanoic acid → butyric acid → acetoacetic acid.

Embden found that the odd-numbered acids formed no acetoacetic acid, which is the result anticipated if these acids also undergo β-oxidation.

We have found, in accordance with Embden, that the rate of production of acetoacetic acid from fatty acids varies in an alternate manner, the even-numbered acids producing much more acetoacetic acid than their odd-numbered neighbours. A new result is that, with guinea-pig liver at least, the odd-numbered fatty acids—apart from formic and propionic acids—also give rise to small but significant amounts of acetoacetic acid.

It is therefore likely that β-oxidation, if it occurs at all, is not the only type of oxidation which takes place with the odd-numbered acids.

The fatty acids produce β-hydroxybutyric acid as well as acetoacetic acid. The hydroxy-acid is probably produced by reduction of acetoacetic acid, for this has been shown to be true in the case of crotonic and butyric acids [Jowett and Quastel, 1935, 1]. The production of total ketones is therefore in all probability the best measure of the gross acetoacetic acid production.

The increase in respiration of guinea-pig liver brought about by odd-numbered acids is greater than that due to their even-numbered neighbours, which suggests that the odd-numbered acids are more completely oxidised. This more complete oxidation would be expected if odd-numbered acids yield a three-carbon acid which is readily oxidised by the liver, in contrast to acetoacetic acid, which is little oxidised.

The yields of total ketone bodies obtained from the even-numbered acids of 4 to 10 carbon atoms with guinea-pig liver are of the order of one molecule of ketone bodies per molecule of fatty acid.

So far, then, we have a number of facts which are in general accord with the β-oxidation theory, although there is a suggestion that the odd-numbered acids possess also another mode of oxidation which may not be quantitatively of great importance. The facts next to be considered may also be interpreted on the β-oxidation theory, if we add to this an assumption regarding the nature of the product formed from the two carbon atoms lost in each stage of oxidation. We have, in fact, found that fixed acid is produced during the oxidation of the higher fatty acids by guinea-pig liver. The acid (or acids) has not yet been identified. If it should be acetic acid, or another 2-carbon acid, our results regarding acid production are in qualitative agreement with the predictions of the β-oxidation theory. These results have already been discussed.

The suggestion that acetic acid is a product of fatty acid oxidation leads to the query whether acetic acid might not be the source of the acetoacetic acid formed by the higher odd-numbered fatty acids. Since these acids form acetoacetic acid at a higher rate than does acetic acid with guinea-pig liver, it is

probable that the explanation does not suffice, and that β-oxidation will not explain the acetoacetic acid production of odd-numbered acids.

Considering more quantitatively the yields of ketone bodies obtained from the even-numbered fatty acids with guinea-pig liver, we see that butyric acid gives a lower yield than do the higher fatty acids. Here, again, we may ask whether the higher yield obtained from the higher acids may not be due to their yielding acetic acid, which may be a source of additional ketone bodies. From our data, we consider that this possible explanation of the higher yields is inadequate. Now, according to the β-oxidation theory, butyric acid may give a higher yield of ketone bodies than do the higher fatty acids but cannot give a lower yield. Taking into account acetic acid as a possible intermediary, we find the position little altered. We, therefore, consider that the facts regarding yields are incompatible with the β-oxidation theory.

A more striking discrepancy still is found in the effect of benzoate on the rates of acetoacetic acid and total ketone body production from the even-numbered fatty acids. The rates are much more strongly inhibited in the case of butyrate than with the higher fatty acids. In the absence of an inhibitor the rates of acetoacetic acid production from the 4-, 6- and 8-carbon acids are approximately equal. In the presence of benzoate the 6-, 8- and 10-carbon acids yield acetoacetic acid and total ketones at definitely higher rates than does butyric acid. Since the production from acetic acid is low, and is as strongly inhibited by benzoate as that from butyric acid, it does not avail to suggest that acetic acid is an intermediary and is causing an apparent discrepancy with the β-oxidation theory. We must therefore conclude that the even-numbered fatty acids are not broken down through butyric acid as an intermediary. Nor, according to our data, can hexanoic acid be an intermediary for the higher acids.

We are therefore forced to abandon the β-oxidation theory. In attempting to formulate a new hypothesis to replace it, we will first point out that at the present stage such a hypothesis must differ from the β-oxidation theory more in the mechanism of oxidation which it proposes than in the nature of the products which it supposes to be formed.

We propose the view that fatty acids undergo, at a common enzyme, an oxidation throughout the fatty chain, alternate carbon atoms being affected. The oxidised product then breaks down. We may term this a theory of "multiple alternate oxidation"[1].

At the present time, the detailed application of this theory is necessarily somewhat speculative. The oxidised intermediary substances, which may exist only in combination with the enzyme, may be supposed to be capable of breaking down in a number of ways.

The theory is illustrated below by the supposition that an even-numbered acid, octanoic acid, undergoes oxidation at its β-, δ- and ζ-carbon atoms before the chain breaks, and that an odd-numbered acid, valeric acid, may undergo either β-oxidation or oxidation at the α- and γ-carbon atoms.

(1) Even-numbered acid:

$$CH_3.CH_2.CH_2.CH_2.CH_2.CH_2.CH_2.COOH$$

$$\rightarrow CH_3.\overset{\vee}{C}.CH_2.\overset{\vee}{C}.CH_2.\overset{\vee}{C}.CH_2.COOH + 6H$$

$$\rightarrow [CH_3.CO.CH_2.CO.CH_2.CO.CH_2.COOH]$$

$$2CH_3.CO.CH_2.COOH \quad \text{or} \quad CH_3.CO.CH_2.COOH + 2CH_3.COOH$$

[1] A somewhat similar view was put forward by Hurtley [1916].

(2) Odd-numbered acid:

(*a*) β-oxidation

$$CH_3.CH_2.CH_2.CH_2.COOH$$

$$\rightarrow CH_3.CH_2.\overset{\vee}{C}.CH_2.COOH + 2H$$
$$\rightarrow [CH_3.CH_2.CO.CH_2.COOH]$$
$$\rightarrow CH_3.CH_2.COOH + CH_3.COOH$$

(*b*) $\alpha\gamma$-oxidation

$$CH_3.CH_2.CH_2.CH_2.COOH$$

$$\rightarrow CH_3.\overset{\vee}{C}.CH_2.\overset{\vee}{C}.COOH + 4H$$
$$\rightarrow [CH_3.CO.CH_2.CO.COOH]$$

$$CH_3.CO.CH_2.COOH + CO_2 \text{ (or } H.COOH) \quad \text{or} \quad CH_3.COOH + CH_3.CO.COOH$$

The detailed working out of our hypothesis is still, as just pointed out, a matter of speculation. We cannot dismiss the possibility that even-numbered fatty acids may undergo oxidation at their α-, γ-, ϵ- ... carbon atoms, or the possibility that ω-oxidation may occur with the higher fatty acids to some considerable extent.

We can do little more now than present the hypothesis that oxidation proceeds along the whole chain of a fatty acid molecule at alternate carbon atoms, with the ultimate formation of acetoacetic acid and other acid products. Oxidation at the β-, δ- ... carbon atoms is considered to be the major process in the oxidation of fatty acids containing four or more carbon atoms.

Summary.

1. An investigation has been made of the formation of acetoacetic and β-hydroxybutyric acids, resulting from the oxidation of the normal saturated fatty acids containing from two to ten carbon atoms, in the presence of rat and guinea-pig liver. Van Slyke's method for estimating ketone bodies is used on a micro-scale.

2. Acetoacetic acid is probably the only β-keto-acid produced in significant quantities by the oxidation of fatty acids.

3. Acids of four, six and eight carbon atoms produce acetoacetic acid most rapidly. The ten-carbon acid is slightly less active and acetic acid considerably less active.

4. Acids of five, seven and nine carbon atoms produce acetoacetic acid at small but significant rates with guinea-pig liver. Propionic acid does not form acetoacetic acid.

5. The greater increase in the respiration of liver brought about by the odd-numbered fatty acids suggests that these are more completely burned to carbon-dioxide and water than their even-numbered neighbours.

6. In the case of guinea-pig liver, about one molecule of ketone bodies is produced per molecule of hexanoic or octanoic acid, and rather less is produced with decanoic acid. The yield of ketone bodies from butyric acid is considerably lower.

7. With all fatty acids higher than valeric acid, oxidation results in the formation of fixed acid, which is greater with the even-numbered fatty acids than with the odd-numbered fatty acids. The nature of the fixed acid produced,

which is neither acetoacetic nor β-hydroxybutyric acid, has not been determined. The oxidation of propionic and valeric acids results in disappearance of fixed acid.

8. Sodium benzoate inhibits the acetoacetic acid and total ketone body production from butyric acid to a greater extent than from the higher fatty acids in the presence of rat liver. The inhibition decreases on ascending the series of even-numbered acids.

9. The results are discussed in relation to the theory of successive β-oxidation, which is shown to be inadequate to explain the facts. A hypothesis of "multiple alternate oxidation" is put forward, according to which the fatty acids are supposed to be oxidised at alternate carbon atoms along the whole length of the chain before breakdown to acetoacetic and other acids takes place.

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Effects of Salicylate and other Benzoates on Oxidative Enzymes of the Tricarboxylic Acid Cycle in Rat Tissue Homogenates[1]

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Received August 20, 1953

Introduction

It has been known for many years that enzymes oxidizing such substrates as succinate, lactate, pyruvate, glucose, and D-amino acids are inhibited by benzoate, salicylate, and various other aromatic carboxylic acids (1–10). On the other hand, Alwall (11) found that serum citrate levels were diminished during salicylate therapy. Since he found (12) that citrate oxidation by rabbit kidney or liver was increased in the presence of salicylate, he attributed the decrease in serum citrate to increased oxidation in the presence of salicylate. If this is so, it seems likely that a study of the effect of salicylate and related compounds on the reactions of the tricarboxylic acid cycle may give some insight into the nature of the inflammatory processes which respond to salicylate therapy.

Experimental

Rat kidney cortex homogenates in 0.15 *M* KCl were used as the enzyme source in most cases. Liver was used in some cases for comparison. Rats were stunned by a blow on the head, exsanguinated by decapitation, and the desired organs removed and chilled. Kidneys were demedullated. The organs were freed of adhering liquid, weighed, and homogenized with KCl in a chilled glass Potter-Elvehjem homogenizer. Homogenates were filtered through muslin before use.

Preliminary experiments indicated that the reaction between inhibitor and enzyme was not instantaneous, and that it was sometimes influenced by the presence of substrate. In most experiments, therefore, homogenate was added to chilled flasks containing all components of the system except substrate. After 30 min. at the temperature of ice water, substrate was added, and the flasks were

[1] This work was supported in part by a grant from the U. S. Public Health Service.

placed in the Warburg bath. All inhibitors were tested at a concentration of 0.0067 *M* unless otherwise noted.

Enzyme activities were determined at 30° with air as the gas phase. Flasks were incubated in the bath 10 min. before stopcocks were closed. Oxygen uptakes were measured at 10-min. intervals. All experiments were run in duplicate, and average values are given in the graphs and tables. Duplicates were in agreement with a precision of at least 10%, considerably better in most cases. All buffers were used as potassium salts; substrates, inhibitors, and cofactors were all neutralized with KOH. When ferricyanide was the ultimate electron acceptor rather than molecular oxygen, the gas phase was 95% nitrogen-5% CO_2, and evolution of CO_2 from an $NaHCO_3$ buffer was measured. No correction was made for CO_2 retention. All results were [illegible]ted for [illegible] enzymes in the absence of added sub-

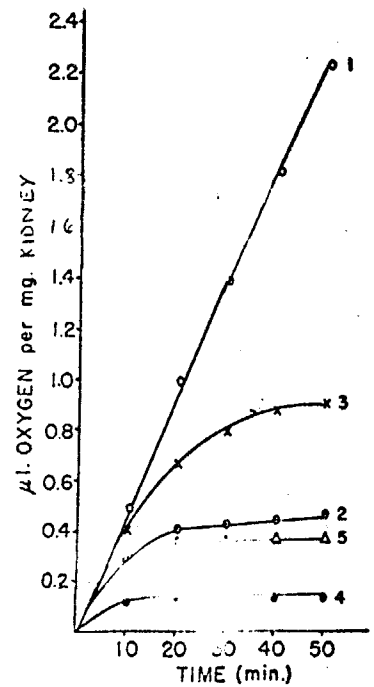


Fig. 1. Oxidation of citrate by rat kidney homogenates. Comparison of phenol and benzoate with salicylate. Each flask contained 0.5 ml. of 10% kidney homogenate in 0.15 *M* KCl, 0.5 ml. of 0.1 *M* phosphate buffer of pH 7.4, 2 µmoles of adenosine triphosphate (ATP), 2 µmoles of $MgCl_2$, 60 µmoles of DL-citrate, 20 µmoles of each inhibitor indicated to be present, and 0.15 *M* KCl to make a total volume of 3.0 ml. NaOH was present in the center wells. Curve 1 shows the activity in the absence of any inhibitor, curve 2 in the presence of salicylate, curve 3 in the presence of benzoate, curve 4 in the presence of phenol, and curve 5 in the presence of equimolar quantities of phenol and benzoate.

strate. Oxygen uptakes and CO_2 evolutions are expressed as microliters of gas/mg. of wet tissue. Details of the various experiments are given as footnotes to the appropriate figures and tables. Although only one example has been given in each case, all experiments were repeated at least twice to give similar results on preparations from different rats. With preparations of this type it has not always been possible to determine whether inhibition was at equilibrium, so the results have been used only to draw qualitative conclusions as to relative effects in simultaneous experiments under comparable conditions.

Results

Oxidation of Citrate

Contrary to the observation of Alwall (12), it was found that in rat kidney homogenates, salicylate decreased the consumption of oxygen by citrate. To determine whether the inhibitory effect of salicylate was caused by any particular group in the molecule or was a property of the molecule as a whole, it was compared with phenol and benzoate. It can be seen from Fig. 1 that the effect of salicylate is intermediate between those of phenol and benzoate and similar in magnitude to a mixture of the two. The effects of mixtures of salicylate with phenol and with benzoate were also found to be nonadditive.

Since α-ketoglutarate is at the stage of oxidation following citrate in the tricarboxylic acid cycle, formation of α-ketoglutarate was measured to determine whether inhibition was greater for the oxidation of citrate or for subsequent steps in the cycle. The ratio of oxygen uptake to α-ketoglutarate formation was markedly decreased in the presence of salicylate, indicating that the effect of salicylate is much greater for the

TABLE I

Effect of Salicylate on the Oxidation of Citrate in the Presence of Arsenite

The experiments were performed as described in the legend to Fig. 1. The duration of the experiments, including the 10-min. temperature equilibration period, was 40 min. Oxygen uptakes were corrected for this equilibration period by assuming the rates were identical to those in the first 10 min. after stopcocks were closed. Oxidation was arrested at the end of this period by transfer of the flasks to an ice bath and addition of trichloroacetic acid to the flasks. α-Ketoglutarate was determined by the method of Friedemann and Haugen (14).

Arsenite concentration $M \times 10^3$	Salicylate concentration $M \times 10^3$	Oxygen absorbed μg. atomic wt.	α-Ketoglutarate formed μmoles	Ratio of O:α-ketoglutarate
0	0	4.5	0.4	10.7
0	6.7	2.3	0.8	2.7
3.3	0	2.1	0.9	3.6
3.3	6.7	2.7	0.9	2.9

oxidation of α-ketoglutarate than for the preceding oxidative step. Therefore, arsenite, which is known (13) to suppress the oxidation of α-ketoglutarate, was added to the system for oxidizing citrate. It is shown in Table I that the decrease due to salicylate in the ratio of oxygen utilized to α-ketoglutarate formed is much lower in the presence of arsenite. Benzoate also decreased this ratio in a comparable manner to salicylate. In a typical experiment the ratio decreased from 12.8 to 5.7.

Von Euler and co-workers (8, 10) found that salicylate was competitive with diphosphopyridine nucleotide (DPN) in the inhibition of glucose and lactic dehydrogenases. However, in this study the inhibition of citrate oxidation by salicylate was not reversed by the addition of DPN, pantothenate, or ascorbate to the reaction mixture or by the presence of large excesses of substrate, magnesium chloride, or adenosine triphosphate (ATP).

Oxidation of α-Ketoglutarate

The results on the oxidation of citrate in kidney homogenates indicated that the effects of salicylates and related compounds were much

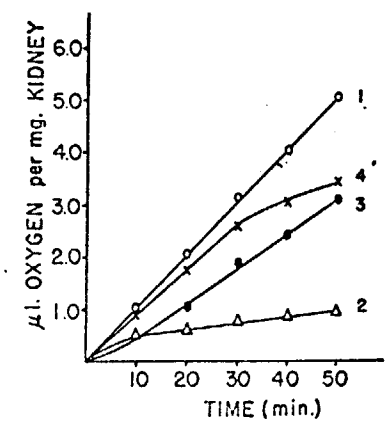


FIG. 2. Effect of salicylate on oxidation of α-ketoglutarate and reversal by $MgCl_2$. Malonate absent. Each flask contained 0.5 ml. of 5% kidney homogenate in 0.15 *M* KCl, 0.5 ml. of 0.1 *M* phosphate buffer of pH 7.4, 3 μmoles of ATP, 0.04 μmoles of cytochrome c, 8 μmoles of $MgCl_2$, 30 μmoles of α-ketoglutarate, 20 μmoles of salicylate when indicated to be present, and 0.15 *M* KCl to make a total volume of 3.0 ml. When excess $MgCl_2$ was present an additional 50 μmoles was added. NaOH was present in the center wells. Curve 1 shows the activity of the control without inhibitor or excess of $MgCl_2$, curve 2 the activity in the presence of salicylate, curve 3 in the presence of excess $MgCl_2$, and curve 4 in the presence of excess $MgCl_2$ and salicylate.

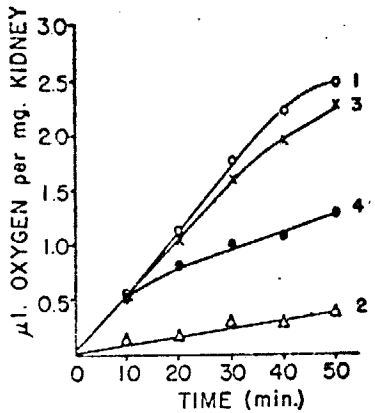


FIG. 3. Effect of salicylate on oxidation of α-ketoglutarate and reversal by $MgCl_2$. Malonate present. This experiment was performed simultaneously with that shown in Fig. 2, using twice the amount of the same homogenate and 10 μmoles of malonate in each flask. Otherwise, the contents were identical. Curves are numbered as in Fig. 2.

greater at the next stage of oxidation in the cycle, oxidation of α-ketoglutarate. In this case oxidation could be limited to a single step by the presence of malonate to inhibit the oxidation of succinate (15). Salicylate inhibited the oxidation of α-ketoglutarate by rat kidney homogenates, whether malonate was present or absent, and it was found that the inhibition was overcome by the presence of a large excess of magnesium chloride (0.019 *M*), as shown in Figs. 2 and 3. This concentration of magnesium chloride often inhibited the oxidation (curve 3, Figs. 2 and 3), but in all cases the inhibition by salicylate in the presence of 0.019 *M* $MgCl_2$ was much lower than in the presence of 0.0023 *M* $MgCl_2$. Activity in the presence of salicylate was frequently greater than in its absence when large quantities of magnesium chloride were present (curves 3 and 4, Fig 2). Analyses for the effect of salicylate on α-ketoglutarate consumption showed no definite trend in the ratio of oxygen to α-ketoglutarate used. However, since ratios less than one were frequently encountered, some of the substrate must have been used in a nonoxidative reaction. Such an effect was observed by Sanadi, Littlefield, and Bock (16) with their preparation of α-ketoglutaric dehydrogenase.

As shown in Table II, phenol, benzoate, and various hydroxybenzoates inhibited the oxidation of α-ketoglutarate, and the activity was largely restored by the presence of an excess of magnesium chloride. Acetylsalicylate had approximately the same effect as salicylate and may have been hydrolyzed to salicylate by the homogenate. In no other case was the reversal of the inhibition by magnesium chloride as complete as for salicylate, and the reversal was much lower for the dihydroxybenzoates than for any of the other inhibitors. The effect of salicylate was similar in magnitude both in the presence and in the absence of malonate. However, the inhibitory effect of phenol was smaller in the absence of malonate and greater in the one-step reaction. The effect of benzoate was much greater when malonate was present than when it was absent. It will be seen later that differences in relative inhibitions in the presence and absence of malonate can be accounted for on the basis of effects of the inhibitors on succinic dehydrogenase when malonate is absent.

Manganous chloride could replace magnesium chloride in the system for the oxidation of α-ketoglutarate, and inhibition occurred when salicylate was added. However, no reversal of inhibition could be demonstrated when an excess of manganese was added.

It was of interest to determine whether the effect of salicylate occurred at the dehydrogenation of substrate or at some later stage in the transfer of electrons to molecular oxygen. Cytochrome oxidase in aqueous rat

TABLE II

Effects of Phenol, Benzoate and Hydroxybenzoates on Oxidation of α-Ketoglutarate

The experiments were carried out as described in the legends to Figs. 2 and 3, each inhibitor being compared with the same concentration of salicylate run simultaneously on the same enzyme preparation. The duration of the experiment was 50 min. after stopcocks were closed, except in Expt. 8 with malonate absent where the duration was 40 min. The relative inhibition was taken as the ratio of inhibition of oxygen uptake by the inhibitor to that by salicylate in the same experiment. The last column gives the per cent of the inhibition which was reversed in the presence of excess $MgCl_2$. In Expt. 5 the inhibition by salicylate was only restored by excess $MgCl_2$ to the extent of 72% compared to 100% in most other cases.

Inhibitor	Relative inhibition: Malonate present	Relative inhibition: Malonate absent	Per cent reversed by excess $MgCl_2$ (malonate absent)
1. Phenol	1.2	0.9	65
2. Benzoate	0.8	0.3	63
3. *p*-Hydroxybenzoate	0.7	0.7	85
4. *m*-Hydroxybenzoate	0.9	0.8	74
5. Gentisate	0.8	1.0	14
6. Salicylate	1	1	100
7. Acetylsalicylate	—	1.0	97
8. γ-Resorcylate	1.1	1.4	37

TABLE III

Effect of Salicylate on Oxidation of α-Ketoglutarate by Rat Liver Homogenates

The experiments were performed as described in the legends to Figs. 2 and 3, except that liver homogenates were used. Duration of the experiments was 40 min. in the absence of malonate, 50 min. in the presence of malonate. Different enzyme preparations were used for the experiments in the presence and absence of malonate, respectively.

$MgCl_2$ concentration $M \times 10^3$	Salicylate concentration $M \times 10^3$	Oxygen absorbed, μl./mg. wet liver: Malonate present	Malonate absent
2.7	0	1.9	1.3
2.7	6.7	2.1	0.9
19.3	0	1.3	1.0
19.3	6.7	2.5	1.5

kidney homogenates was not affected by salicylate when assayed by the method of Schneider and Potter (17). When α-ketoglutarate was oxidized by rat kidney homogenates in isotonic potassium chloride with ferricyanide as the electron acceptor, the cytochrome system being bypassed, oxidation was still inhibited by salicylate. In an experiment in which carbon dioxide evolution was measured for 30 min., gas formation in the presence of salicylate was only 17 % of that in the absence of inhibitor.

In the absence of malonate, salicylate also inhibited oxidation of α-ketoglutarate by rat liver homogenates. Although either salicylate or an excess of magnesium was inhibitory, it can be seen from Table III that when they were both present, the activity was greater than that of the control containing the optimal quantity of magnesium and no inhibitor. In the presence of malonate, salicylate did not inhibit liver α-ketoglutaric dehydrogenase, but prevented inhibition by excess magnesium.

Oxidation of Succinate

It was possible to carry out the oxidation of succinate through the tricarboxylic acid cycle in the presence of magnesium ions and ATP; or, by using water homogenates and the system of Schneider and Potter (17), the reaction could be limited to a single step. In either case the reaction was inhibited by 0.0067 *M* salicylate, the level used to inhibit oxidation of citrate and of α-ketoglutarate. However, unlike the inhibition of α-ketoglutaric dehydrogenase, this inhibition was not reversed by the presence of an excess of magnesium chloride. The results are shown in Figs. 4 and 5. Water homogenates fortified with cytochrome were more

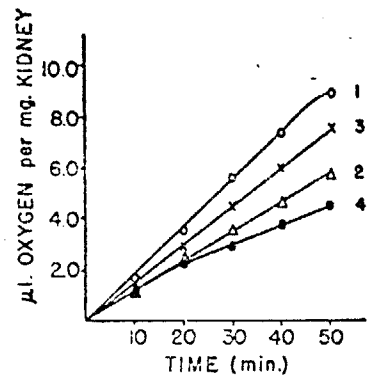


FIG. 4. Effect of salicylate on oxidation of succinate, KCl homogenate. Each flask contained 0.5 ml. of 5% kidney homogenate in 0.15 *M* KCl, 0.5 ml. of 0.1 *M* phosphate buffer of pH 7.4, 1 μmole of ATP, 2 μmoles of $MgCl_2$, 100 μmoles of succinate, 20 μmoles of salicylate when indicated to be present, and 0.15 *M* KCl to make a total volume of 3.0 ml. When excess $MgCl_2$ was present, an additional 50 μmoles was added. NaOH was present in the center wells. Curves are labeled as in Fig. 2.

active than isotonic salt homogenates, probably because of the presence of calcium chloride. Calcium ions prevent the oxidation of fumarate to oxalacetate, which is inhibitory to succinic dehydrogenase (17).

As in the case of α-ketoglutaric dehydrogenase, when ferricyanide was the ultimate electron acceptor, the oxidation was inhibited by salicylate. For example, in an experiment of 40 min. duration with ferricyanide as the oxidant, evolution of carbon dioxide in the presence of salicylate was about 80 % of that of the uninhibited control in each of the four intervals during which activity was determined. The effect of salicylate, therefore, occurred at a step preceding the cytochrome system in the chain of electron transfer.

The relative inhibitory effects of phenol, benzoate, and various hydroxybenzoates are shown in Table IV. The one-step oxidation of succinate and oxidation under conditions where the entire tricarboxylic acid cycle can operate are included. Relative inhibitions did not correspond to those observed for α-ketoglutaric dehydrogenase. The effect of salicylate was much greater than either that of phenol or of benzoate. Relative inhibitions were similar for both the water and isotonic saline homogenates.

The stability of succinic dehydrogenase in water homogenates made it possible to examine the inhibition for reversibility by the criteria of Ackermann and Potter (18). For any concentration of inhibitor, if the

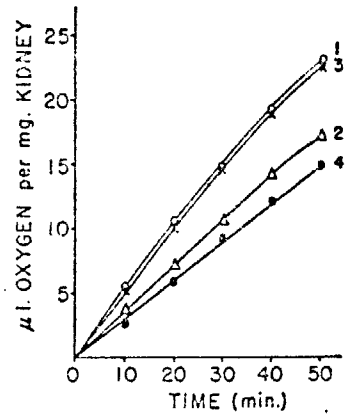


FIG. 5. Effect of salicylate on oxidation of succinate. Water-homogenate. Each flask contained 0.5 ml. of 5% kidney homogenate in glass-distilled water, 1.0 ml. of 0.1 *M* phosphate buffer of pH 7.4, 1.2 μmoles each of $CaCl_2$ and $AlCl_3$, 0.16 μmoles of cytochrome c, 150 μmoles of succinate, 20 μmoles of salicylate when indicated to be present, and water to make a total volume of 3.5 ml. When $MgCl_2$ was present, 50 μmoles was added to the system. NaOH was present in the center wells. In this case the homogenate was made with distilled water and no precautions were taken to keep the temperature below room temperature. The incubation of homogenate for 30 min. with inhibitor and other components of the system before adding substrate was also carried out at room temperature. Curve 1 shows the activity in the absence of inhibitor or added $MgCl_2$, curve 2 in the presence of salicylate, curve 3 with added $MgCl_2$, and curve 4 in the presence of salicylate and added $MgCl_2$.

plot of enzyme activity against enzyme concentration is a straight line through the origin, the inhibition is reversible. The data of Fig. 6 indicate that this is the case. However, Lineweaver-Burk plots were not simple curves, so inhibition constants could not be determined. Inhibition was not reversed by the presence of an excess of substrate or of any of the cofactors added to the system. Addition of ATP, biotin, ascorbic acid, folic acid, pantothenic acid, or bovine plasma albumin also did not reverse the inhibition.

Salicylate did not inhibit succinic dehydrogenase in liver homogenates in isotonic potassium chloride. However, with water homogenates liver gave similar effects to kidney homogenates (Table V). In the isotonic homogenate experiments there was an inhibitory effect of magnesium which was reversed by salicylate. This inhibitory effect was not reversed to any extent in the one-step reaction.

TABLE IV

Effects of Phenol, Benzoate and Hydroxybenzoates on Oxidation of Succinate

The experiments were performed as described in the legends to Figs. 4 and 5, each inhibitor being compared with the same concentration of salicylate run simultaneously on the same enzyme preparation. Duration of the experiments was 50 min. after stopcocks were closed. Relative inhibition was taken as the ratio of inhibition of oxygen uptake by the inhibitor to that by salicylate in the same experiment.

Inhibitor	Relative inhibition: Water homogenate	Relative inhibition: KCl homogenate
1. Phenol	0.2	0.4
2. Benzoate	0.3	0.5
3. *m*-Hydroxybenzoate	0.1	0.1
4. *p*-Hydroxybenzoate	0.2	0.2
5. Gentisate	2.5	2.2
6. Salicylate	1	1
7. γ-Resorcylate	1.5	1.5

Oxidation of Malate

The oxidation of malate did not occur to any appreciable extent in kidney homogenates under the conditions used in these experiments, probably because the oxalacetate produced has an inhibitory effect upon the reaction (19). However, oxidation occurred if pyruvate was present,

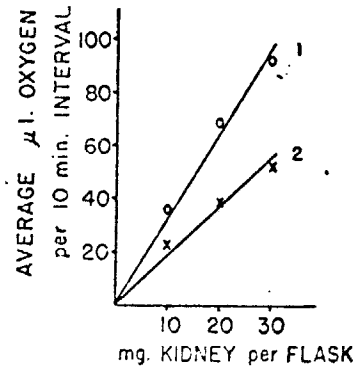


FIG. 6. Effect of enzyme concentration on inhibition of oxidation of succinate. The experiment was carried out as described in the legend to Fig. 5 except that succinate was placed in the side arms of the Warburg flasks and tipped into the system after 30 min. incubation in the bath at 30°. Enzyme was added as a 10% homogenate, 0.1 ml., 0.2 ml., and 0.3 ml. being added to give the three points indicated on each curve. The total volume was 3.0 ml. Duration of the experiment was 50 min. Curve 1 shows the activity in the absence of inhibitor, curve 2 in the presence of salicylate.

TABLE V

Effect of Salicylate on Oxidation of Succinate by Rat Liver Homogenates

The experiments were carried out as described in the legends to Figs. 4 and 5, except that liver homogenates were used. Duration of the experiments was 50 min.

	$MgCl_2$ concentration $M \times 10^2$	Salicylate concentration $M \times 10^3$	Oxygen absorbed *µl./mg. wet liver*
A. Water homogenate	0	0	13.6
	0	5.7	7.7
	14.3	0	11.2
	14.3	5.7	6.6
B. KCl homogenate	0.7	0	3.5
	0.7	6.7	3.4
	17.4	0	2.8
	17.4	6.7	3.8

although pyruvate was not oxidized by kidney homogenates in the absence of malate. The effect of salicylate upon the oxidation of malate is shown in Fig. 7. In most experiments the initial inhibition by salicylate was fairly small, but inhibition increased rapidly during the experiment and was essentially complete after about 40 min. The system of Potter (20) was therefore examined for the effect of salicylate. In this system,

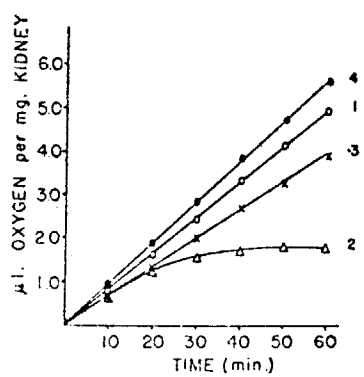
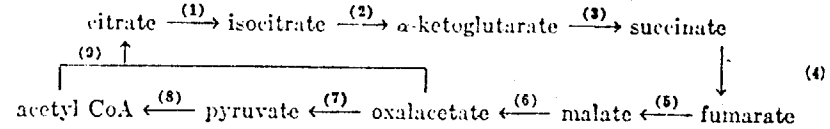


FIG. 7. Effect of salicylate on oxidation of malate and reversal by $MgCl_2$. Each flask contained 0.5 ml. of 5% rat kidney homogenate in 0.15 *M* KCl, 0.5 ml. of 0.1 *M* phosphate buffer of pH 7.4, 3 µmoles of ATP, 8 µmoles of $MgCl_2$, 10 µmoles of pyruvate, 20 µmoles of DL-malate, 20 µmoles of salicylate when indicated to be present, and 0.15 *M* KCl to make a total volume of 3.0 ml. When excess $MgCl_2$ was present, an additional 50 µmoles was added. NaOH was present in the center wells. Curves are numbered as in Fig. 2.

which requires no magnesium, oxidation by an aqueous homogenate is limited to a single step by the presence of glutamate to transaminate with oxalacetate as it is formed. Salicylate had little or no effect upon this system in both kidney and liver homogenates.

Discussion

The foregoing experiments indicate that salicylate inhibits the tricarboxylic acid cycle at a minimum of two of the steps (steps 3 and 4), shown in the accompanying diagram. Other steps may be inhibited to a lesser extent.



For example, the experiments on inhibition of citrate oxidation indicate that the effects of salicylate and benzoate are more intense at a later stage of the cycle, but inhibition of aconitase (step 1) or of isocitric dehydrogenase (step 2) may occur to a small extent. Similarly, there may be some inhibition of malic dehydrogenase (step 6) although the measurements would indicate that the effect of the inhibitors tested is much greater at some other stage of the cycle, possibly oxidation of pyruvate (step 8) or condensation of the product with oxalacetate (step 9).

It is possible that magnesium is required by α-ketoglutaric dehydrogenase (step 3) in order to bind the substrate to the enzyme in the manner suggested by Smith and Lumry (21). In the presence of salicylate or other inhibitors, the magnesium is tied up to an extent depending on the affinity of the inhibitor for magnesium, and substrate cannot be bound by the enzyme. An excess of magnesium permits binding of the inhibitor with sufficient metal ions remaining to bind substrate to enzyme. In the presence of a sufficient excess of magnesium the enzyme is inhibited, doubtless because of the ability of the unbound magnesium ions to bind substrate and make it unavailable to the magnesium bound on the enzyme. By binding this excess of magnesium, salicylate can therefore enhance the activity of the enzyme for substrate, as was observed in many cases. It is possible that in Alwall's experiments (12), sufficient magnesium was present to permit this phenomenon to occur. It is noteworthy that the enzyme of Sanadi *et al.* (16), which does not require added magnesium (22), is not inhibited by salicylate. It is not known

whether this preparation contains a small quantity of magnesium, which might be sufficient to saturate the enzyme. However, since the enzyme is from a different animal and tissue (pig heart), it may differ from that in rat kidney and liver.

While manganese can replace magnesium, the inhibition by salicylate is not reversed by the presence of excess manganous chloride. It may be that manganese has a greater affinity for the enzyme than magnesium and remains bound even in the presence of salicylate. If salicylate is then bound to the enzyme through the manganese, it can prevent the access of substrate.

The effects in the presence of malonate may be attributed largely to a one-step reaction (step 3), whereas in its absence all the reactions of the tricarboxylic acid cycle may be assumed to occur at least to some extent. However, it should be kept in mind that malonate and magnesium ions are antagonistic (23) and that at least part of the apparent reversal of inhibition by excess magnesium ions may consist in permitting further reactions in the cycle to occur.

In liver homogenates the situation is apparently more complicated. Sufficient magnesium may be present in the homogenates to permit one-step oxidation of α-ketoglutarate to occur at the maximum rate even when salicylate is added. However, when the entire cycle is operative, magnesium becomes limiting if salicylate is present.

The relative effects of various inhibitors in step 3 are probably related to their affinity for magnesium, although other factors are doubtless involved. Salicylate, gentisate, and γ-resorcylate, which are capable of forming chelates, probably have the greatest affinity for magnesium, and their inhibitory effect is greatest. With salicylate the reversal of inhibition by magnesium is essentially complete, but this restoration of activity is much lower for the dihydroxybenzoates. In these cases, even when magnesium is bound in a chelate structure, there is still a residual phenolic group which may either be bound to magnesium or attack the enzyme at some other active center.

While the effect of phenol upon α-ketoglutaric dehydrogenase is greater than that of salicylate, its effect on succinic dehydrogenase is much lower. The relative effects of benzoate and the monohydroxybenzoates (other than salicylate) are greater for α-ketoglutaric dehydrogenase than for succinic dehydrogenase. These facts would support the conclusion that the two enzymes are attacked at active centers of different character. In the case of α-ketoglutaric dehydrogenase, all the inhibitors probably

attack the same active center, at least in part, as they are all at least partially antagonistic to magnesium. On the other hand, for succinic dehydrogenase, which does not require magnesium, it is possible that phenol and benzoate, for example, are sufficiently different chemically that they attack different active centers in the enzyme molecule. This may account in part for the variation in relative activity.

The inhibition of succinic dehydrogenase by salicylate shows characteristics of reversibility, but activity has not been restored by any substance tested. Since there is no effect of salicylate upon cytochrome oxidase, and inhibition occurs with ferricyanide as the oxidant, it is possible that salicylate interferes reversibly with the binding of some factor such as the Slater factor (24) or a flavin coenzyme. If so, this factor is probably present in much higher concentration in liver homogenates than in kidney homogenates, but is either dissociated or destroyed in the preparation of aqueous homogenates.

Summary

Salicylate and related compounds inhibit the Krebs cycle in kidney and liver homogenates. The oxidative enzymes inhibited to the greatest extent are α-ketoglutaric dehydrogenase and succinic dehydrogenase. Inhibition of the former is reversed by the presence of an excess of magnesium ions. The two enzymes also differ in the relative inhibitory effects of other substances chemically related to salicylate. Inhibition of oxidation of succinate is also reversible, but the factor which reverses this inhibition is unknown. When magnesium is present in excess, salicylate may enhance oxidative activity.

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The Journal of Biochemistry, Vol. 43, No. 2, 1956

REACTION OF VERDOHEMOGLOBIN FORMATION IN VITRO, WITH SPECIAL REFERENCE TO ITS QUANTITATIVE TREATMENT

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(Received for publication, January 11, 1956)

In our previous investigation (*1*), it was proved that the breakdown of heme in the system of hemoglobin(or myoglobin)—ascorbic acid—oxygen leading to the formation of verdohemoglobin (the immediate precursor of biliverdin) occurs in the following sequence:

Hemoglobin (or myoglobin)→choleglobin→verdohemoglobin (or verdomyoglobin)

A hypothesis for the reaction mechanism was also presented in the same work. As was then pointed out, verdohemoglobin, the immediate precursor of biliverdin, was demonstrated for the first time spectroscopically in this reaction system.

In the present report, the said reaction was quantitatively investigated mainly through the use of myoglobin as the starting material instead of hemoglobin. The reaction proceeds more smoothly in the case of myoglobin compared with that of hemoglobin, since there occurred no choleglobin-b formation in this system.

As reported by Kikuchi and Tomimura (*2*), the breakdown of hemoglobin in this reaction proceeds more rapidly when benzoate or salicylate was added to the reaction system, these reagents being effective as the "perturbator" to the protein moiety of hemoglobin. This holds also for the reaction with myoglobin and the yield in the verdohemoglobin was found to be significantly increased in this case. This seems to be of great importance to the present attempt to follow up the reaction sequence quantitatively, starting with myoglobin instead of hemoglobin.

EXPERIMENTAL

Material: Metmyoglobin was prepared in a crystalline form from equine heart by the method described by Tsushima and Okazaki (*3*). Spectrometric measurement was carried out by a photoelectric spectrophotometer Model EPB-V of Hitachi Co.

I. Effect of Benzoate upon the Reaction of Verdomyoglobin Formation

Breakdown of heme in the reaction system of myoglobin+ascorbic acid+oxygen was greatly promoted by the addition of sodium benzoate in a suitable concentration (Fig. 1). It is especially notable that the extinction at 760 mμ (specific to verdoheme) was found to be significantly higher than in the system without benzoate (cf. Fig. 3 in the

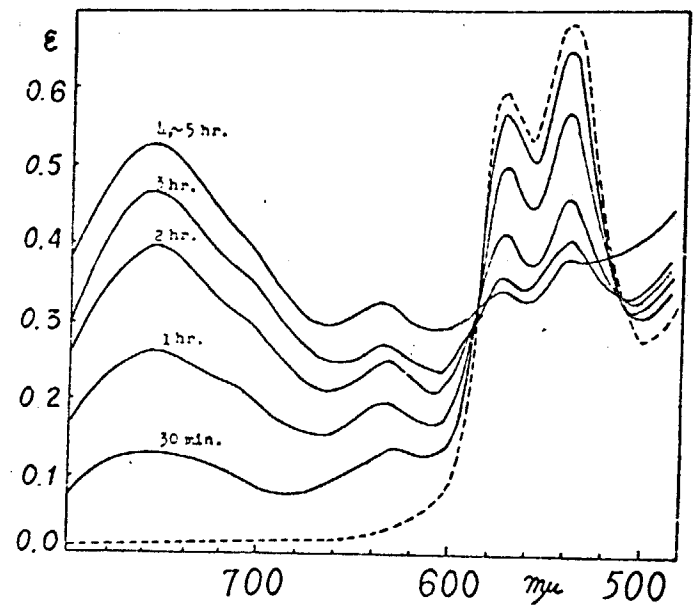

Fig. 1. Absorption change during the reaction process of verdomyoglobin formation in the presence of sodium benzoate.
Metmyoglobin, 2×10^{-4} *M*, 2.5 ml.
Ascorbic acid, 0.1 *M*, 0.5 ml.
Sodium benzoate, 5×10^{-1} *M*, 0.25 ml.
T=37°, pH 7.0

Absorption of reaction solution was measured after addition of 5 ml. 2% NaOH and a small amount of powdered $Na_2S_2O_4$, followed by saturation with CO gas.

previous communication (*1*)). This indicates that the reaction proceeds much more smoothly with the addition of benzoate.

A series of similar experiments were conducted with benzoate in varying concentrations and the absorption spectra of the reaction solutions were measured after 3 hours. Applying the equations established in the previous report to these absorption values found, the extinction of each component present in the reaction solution, namely of protoheme, choleheme, and verdoheme (x, y and z, respectively) was calculated and the values so obtained were plotted against log concentrations of benzoate added. This relationship is shown in Fig. 2. The rate of protoheme decomposition increased with the increasing concentration of benzoate added and in parallel with the increase in the rate of choleheme and verdoheme production. However, when the concentration of benzoate became excessibly higher, the protoheme decomposition became slower.

Under the same experimental conditions as shown in Fig. 2, the oxygen uptake was manometrically measured by the Warburg technique.

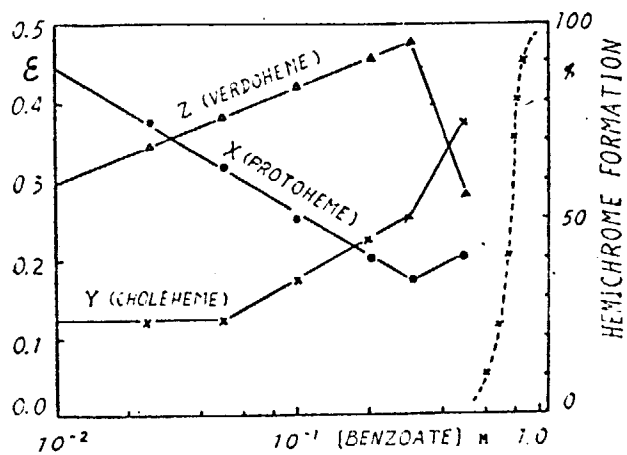

FIG. 2. Changes in the extinction of protoheme, choleheme, and verdoheme (x, y, and z, respectively) in the reaction solutions after 3 hours under the presence of benzoate in varied concentrations.

Reaction conditions and the procedure of the measurement are the same as that given in Fig. 1.

The broken line indicates the relationship between the hemichrome formation (%) from methemoglobin and the concentration of benzoate.

The rate of oxygen uptake increased with the increase of benzoate concentration (Fig. 3). At an optimal concentration range of benzoate the acceleration reached the maximum and remained constant. Above this limit concentration of benzoate, the rate of oxygen uptake again decreased. The benzoate concentration at which the oxidase activity of metmyoglobin began to decrease corresponded to that in which the spectroscopically visible degree of perturbation began to take place in the molecule of metmyoglobin as shown in Figs. 2 and 3 with broken line. The relations represented in Figs. 2 and 3 are qualitatively the same as those which Kikuchi and Tomimura have previously demonstrated in similar experiments using hemoglobin.

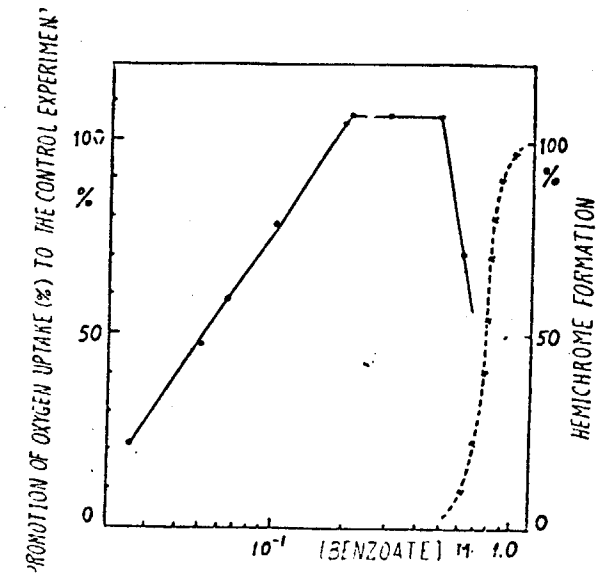

FIG. 3. Promotion of oxidase activity of myoglobin through the effect of sodium benzoate.

O_2-Uptake was measured during 30 minutes with increasing benzoate concentrations.

The promotion of oxidase activity is expressed in percentage increase of O_2-uptake to the control experiment without benzoate.

The addition of salicylate and dodecylsulfate caused an effect similar to that of benzoate. The concentrations of the perturbators, however, had to be higher to be effective to myoglobin than to hemo-

globin. This seems to be due to the stronger resistance of protein moiety of myoglobin than that of hemoglobin against denaturation in general.

II. Determination of Molar Extinction of Verdoheme

The optical constants of protoheme, choleheme and biliverdin have so far been well determined as shown in Table I. As for verdoheme, its spectroscopical constant has not been accurately determined as yet. Based on the experimental results shown in Fig. 1, the molar extinction coefficient could be determined in the following manner.

From the extinctions ε_{573}, ε_{630} and ε_{760} obtained in Fig. 1, the actual increase and decrease of the individual reaction components, protoheme (x), choleheme (y) and verdoheme (z) during the reaction process can be determined by applying the empirical equations presented in our previous paper (*1*). The changes in the extinction of these individual components (x, y and z) during the reaction process could thus be demonstrated as shown in Fig. 4. Among these, the molar concen-

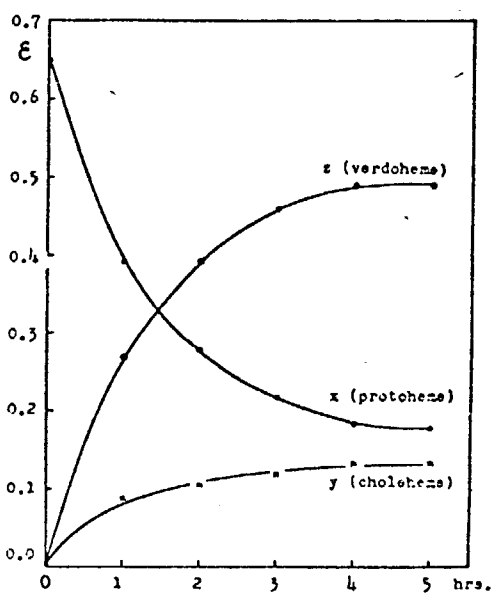

FIG. 4. Changes in the extinctions of protoheme, choleheme, and verdoheme during the course of the reaction.

trations of only protoheme and choleheme can be calculated by applying the constants given in Table I and shown in Fig. 5. If the reaction proceeds without any of the side-reactions, the concentration of verdoheme should be given as follows:

Verdoheme = total heme started − (protoheme remained + choleheme formed)

Now, the concentrations of verdoheme obtained from this equation were plotted against the optical density of the respective z component at 760 mμ (Fig. 6). As shown in Fig. 6, this relation was a straight line indicating that the reaction proceeded without any side reaction as assumed above. By the extrapolation of this straight line in Fig. 6, the molar extinction of verdoheme at 760 mμ was determined as 1.7×10^4. Introducing this value to x component found in the reaction solution (Fig. 4), its absolute concentration was obtained as shown in Fig. 5. Thus it became possible to follow up the whole process of heme breakdown quantitatively in the present reaction system under any of the conditions described.

From the study of the whole process of each component shown in Fig. 5, it became without doubt that the reaction proceeded in the following sequence: Protoheme→choleheme→verdoheme.

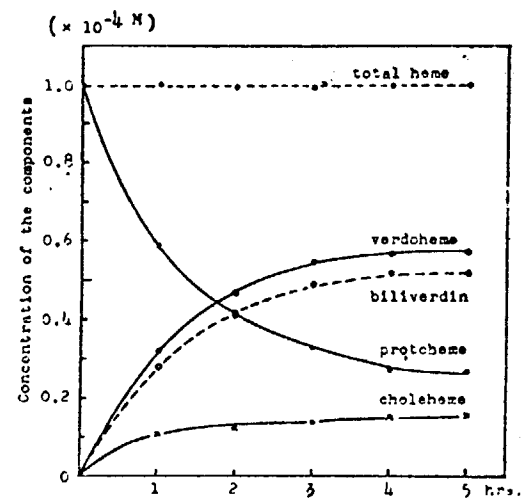

FIG. 5. Changes in each of the components in the reaction solution expressed in terms of absolute concentration. (Calculated from Fig. 4).

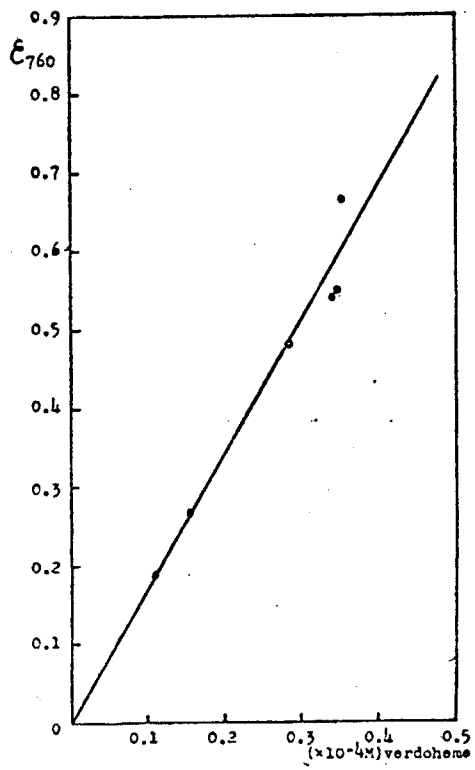

FIG. 6. Calibration curve for verdoheme.

Applying a similar calculation to Fig. 8 in the previous paper, each of the reaction components during the reaction process without the presence of benzoate was obtained as their absolute concentrations (Fig. 7).

III. The Relation between the Amount of Verdoheme Produced and the Yield of Biliverdin

A series of experiments were conducted under conditions similar to that described in Fig. 1 and the verdohemoglobin produced was converted by acid to biliverdin which was then extracted with ether from the reaction solution by the method described in our previous report. The yield of biliverdin was followed up with reaction time. The course of the process is indicated by broken line in Fig. 5. The curve

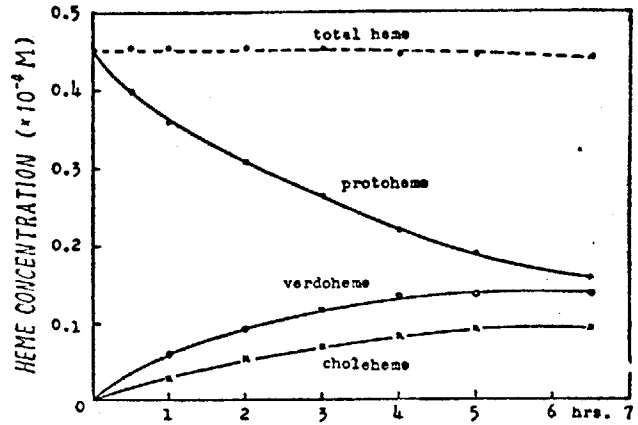

FIG. 7. Changes in each reaction component during the reaction process without benzoate.
(The reaction condition and the procedure of the measurements are the same as those described in Fig. 3 of the previous paper).

of biliverdin obtained agreed fairly well with that of increase in verdoheme, but not with that of choleheme. It became thus evident that the immediate precursor of biliverdin is verdohemoglobin and not choleglobin.

IV. Effect of Ascorbate Concentration upon the Reaction of Verdohemoglobin Formation

Using myoglobin as the starting material and in the presence of benzoate, the reaction was carried out with the addition of ascorbate in varying concentrations. The rate of protoheme breakdown increased with the increasing concentration of ascorbate and in parallel with the increase in the rate of verdoheme formation (Fig. 8). In lower concentrations of ascorbate, the reaction was interrupted more or less at the earlier stage and this is due to the consumption of ascorbate. When ascorbate concentration was estimated after 5.5 hours in the deproteinized reaction solution by the iodine method, the remaining ascorbate was found to be significantly small, as indicated in Table II. In the reaction described in Table II, the initial concentrations of ascorbic acid were taken as 40, 60, 100 and 150 times greater than the concentration of myoglobin. The calculated consumption of as-

corbic acid per mole of decomposed protoheme is shown in the same Table. As seen in this table, about 80–100 moles of ascorbic acid were consumed per mole of protoheme broken down. This value is much higher than that in the reaction with hemoglobin, in which the value was calculated as about 20 moles (*4*). This seems to be due to the lower rate of breakdown process of myoglobin compared with that of hemoglobin.

TABLE I

Spectrophotometric Constants of Protoheme, Choleheme, and Biliverdin

	absorption max., mμ	εmM
Alkali-denatured globin protohemochrome (*4*)	557	30.6
Alkali-denatured globin cholehemochrome (*5*)	618	13.6
CO-compound of protomyoglobin (*3*)	573	12.75
CO-compound of cholemyoglobin	630	16.0
Biliverdin-HCl (*6*)	665	28.6

TABLE II

Initial concentrations of ascorbic acid (*mg./ml.*)	Ascorbic acid / Myoglobin	Ascorbic acid remaining after 5 hrs. (*mg./ml.*)	Protoheme decomposed after 5 hrs. ($\times 10^{-4} M$)	Ascorbic acid consumed / Protoheme decomposed
2.64	150	1.837	0.39	116
1.76	100	0.844		139
		1.056	0.375	106
1.056	60	0.528	0.36	82
0.704	40	0.1425		112
		0.264	0.285	87

V. Experiment with Hemoglobin

In comparison with the reaction starting from myoglobin, a series of experiments were conducted with hemoglobin. Changes of the components in the reaction solution, namely, of protoheme,

choleheme, verdoheme and biliverdin (obtained by acid extraction) were recorded in absolute concentrations. Figs. 9 and 10 are the results obtained in the presence and absence of benzoate, respectively.

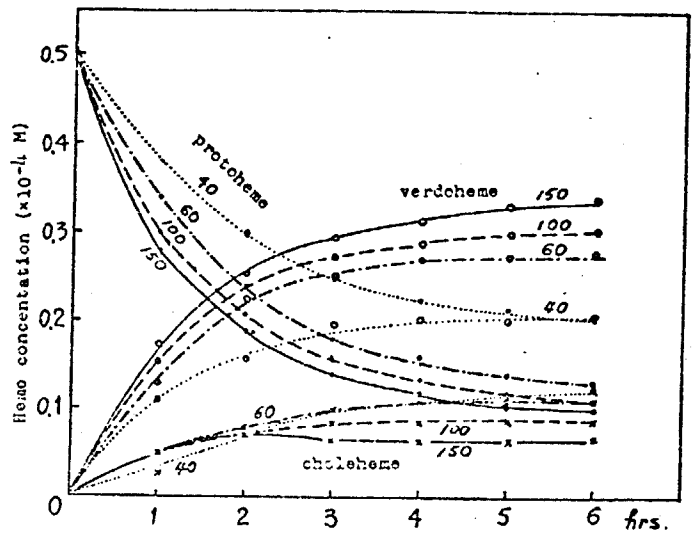

FIG. 8. Changes in each component in the reaction solution with the changes in ascorbate concentration.
Myoglobin $2 \times 10^{-4} M$ 2.5 ml.
Sodium benzoate 0.5 *M* 2.0 ml.
Ascorbic acid (neutralized) 0.5 ml.
T=37°, pH 7.0
The numbers denoted in the figure means the relative multiple of the ascorbate concentration against the concentration of protoheme.

What is common to Figs. 9 and 10, in contrast to the reaction with myoglobin, is a remarkably low production of verdoheme component in spite of a marked increase in choleheme component. In accord with this finding, the yield in biliverdin extractable from this solution was also found to be very low. This is, as described in the previous paper (*5*), due to the intermediate formation of choleglobin-b in the case of the reaction with hemoglobin instead of myoglobin. It is notable, in comparing Fig. 10 with Fig. 9, that choleheme component showed a still higher value and that verdoheme and the yield in biliverdin remained far lower. This indicates that the addition of benzoate

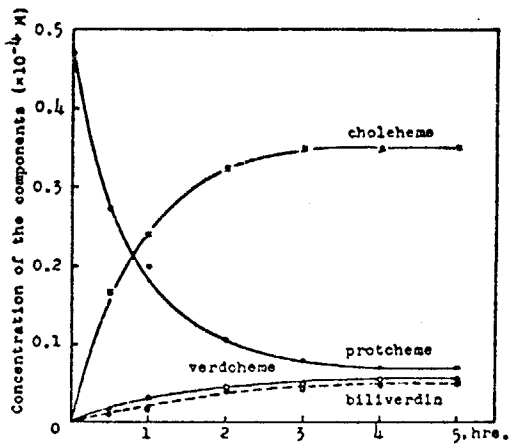

FIG. 9. Changes in each component in the reaction solution starting from hemoglobin.

The absorption was measured after the addition of 12 ml. 1% NaOH and a small amount of powdered $Na_2S_2O_4$, and at each time interval.

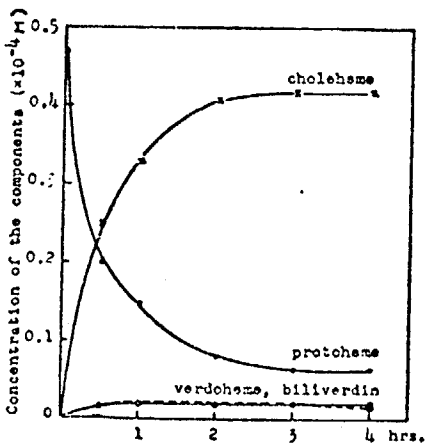

FIG. 10. Hemoglobin breakdown in the presence of benzoate.
Hemoglobin, 3×10^{-4} *M* 2.5 ml.
Ascorbic acid, 0.1 *M* 0.5 ml.
Sodium benzoate, 1.0 *M* 2.0 ml.
T=37°, pH 7.0

is effective in suppressing the conversion of choleglobin into verdohemoglobin. This is due to the promoted formation of choleglobin-b in the presence of benzoate.

It has been reported by Kajita (*6*) in our laboratory that benzoate affects hemoglobin to the unfolding of the molecule (perturbation in the meaning of Holden (*7*)) so that the SH groups in the inner part of the molecule may become exposed. In the reaction system with benzoate added the choleheme produced would be more easily accessible to thus exposed SH groups in the protein moiety to form choleglobin-b. Through the results shown in Fig. 10, the assumption (*8*) that choleglobin-b is the compound of choleglobin combined with protein-SH seems to have gained further support.

DISCUSSION

I. Possibility of Side-reaction

In each of the Figs. 5 to 10, additional data have been given for the total value of heme, the sum of the each component, protoheme, choleheme, and verdoheme. The value of the total heme at every time interval thus given in each of the figures agreed fairly well with the initial value of protoheme. After a longer duration of reaction time (over 7–8 hours), however, the total heme concentration always decreased from the level of initial protoheme. The values of total heme have shown a tendency to decrease after 2 hours, especially in the case of myoglobin reaction and without benzoate (Fig. 7). Some kind of a side-reaction may have occurred at that time.

The most probable side-reaction occurring in this system would be a further decomposition of verdohemoglobin, in whose molecule the porphyrin moiety is converted into biliverdin structure. This possibility may be supported by the following findings. After a longer time intervals, free biliverdin has been detected in the reaction solution as reported in the previous paper. Further, verdohematin prepared from verdohemochrome is stable even in the air, so far as it is kept alkaline. In the neutral solution, however, it undergoes autoxidative decompositions and a pink-colored substance could then be extracted after a treatment with hydrochloric acid. In most cases, the reaction of verdohemoglobin is carried out in a nearly neutral solution so that it seems probable that further oxidation takes place after a longer time intervals. In accord with this assumption, when the reaction was carried out longer,

a pink colored substance could be detected by the procedure giving free biliverdin from the reaction mixture.

The second possible side-reaction is that by detachment of iron atom from choleheme, a new porphyrin which might be termed "choleporphyrin" may form in the reaction solution. For instance, when the reaction solution, starting from myoglobin and after a longer reaction time, was treated with glacial acetic acid followed by shaking with ether to extract biliverdin and verdoheme, the remaining water phase was proved to be Gmelin-positive. On the boundary surface of the solution in contact with nitric acid solution, a blue color of biliverdin could distinctly be observable. In this case, the blue color appeared more strikingly on the contact surface, differing from ordinary Gmelin reaction of bilirubin. Possibly the porphyrin, in which its α-methene has been partially oxidized followed by the detachment of iron atom, is further oxidized through Gmelin reaction at the oxidized α-methene group so that the ring structure is cleaved open. A similar color reaction was observed also with choleglobin-b, assumed to have a similar structure as that of porphyrin now in discussion, obtainable from the reaction solution starting from hemoglobin.

II. Reaction Process to Verdohemoglobin from Choleglobin

It is to be noted in Fig. 8 that, when the initial concentration of ascorbic acid was low, so that the reaction was interrupted rather in the earlier stages, the component choleheme formed was found to be relatively higher in value. From this, it may plausibly be assumed that the process from choleglobin to verdohemoglobin also requires the presence of ascorbate. In an earlier report (*9*), dealing with the reaction of verdohemochrome formation from pyridine hemin under similar conditions as in the present reaction, it has been proved that an intermediate product 630-substance underwent decomposition to verdohemochrome (an immediate precursor of biliverdin) consuming molecular oxygen without the coexistence of ascorbate.

As indicated above, however, the reaction from choleglobin to verdohemoglobin seems to proceed under the participation of a reducing substance such as ascorbic acid. Thus, a more precise examination is required for a further elucidation of the reaction mechanism in this stage.

SUMMARY

By the use of the method described in the present paper, it became possible to follow up quantitatively the reaction process of the formation of verdohemoglobin from hemoglobin or myoglobin through an intermediate choleglobin. The following summarizes the present investigation.

1. The decomposition of myoglobin is promoted by the addition of perturbators as in the case of hemoglobin reaction.
2. The molecular extinction coefficient of verdoheme was determined as $\varepsilon_{mM}^{600}=1.7\times10^4$, in the state of alkali-denatured globin verdohemochrome.
3. The formation of verdoheme and the yield in biliverdin were found to increase with reaction time in parallel.
4. The reaction of verdohemoglobin formation from myoglobin is promoted by increasing concentrations of ascorbic acid added. In the present reaction, 80–100 moles of ascorbic acid was consumed per mole of myoglobin-heme decomposed.
5. Some side-reactions occur after a longer reaction time.
6. The formation of choleglobin-b during the reaction of verdohemoglobin formation starting from hemoglobin increases when perturbators such as benzoate is present in the reaction system. Accordingly, the formation of verdoheme and consequently the yield in biliverdin were found to decrease in the system mentioned.

The present work was aided by the Grant in Aid for Scientific Research from the Ministry of Education, for which our gratitude is due.

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THE TOLERABILITY OF BENZOIC ACID IN CHRONIC FEEDING EXPERIMENT

by W. KIECKEBUSCH and K. LANG

For quite a while benzoic acid has been used as preservative in the preparation of food. On the long term tolerability of the substance only unsatisfactory information is at hand in the literature. A survey and critical evaluation of it as well as a discussion of the tolerability of benzoic acid was published recently by LANG (8).

To fill the gap , we undertook a feeding experiment with benzoic acid stretched over 4 generations in which 2 generations were observed for the whole of their life and whose outcome will be reported as follows:

METHOD

Used were compounds of benzoic acid and sodium benzoate p.a.
The food mixtures were prepared in a food mixer made of steel. As experimental animals served 40-50 gr. rats from a strain left to us by the Paints firm of Bayer from Elberfeld. They were kept in double cages with a simplified form of feeding apparatus of the Colombus one (3). In the first 8 weeks they were fed according to the "paired fed" technique, later ad libitum. The animals always received water ad libitum.

The diet consisted in the commercial rats standard food of the firm Latz (Euskirchen). The controls received none, group 1 0.5% and group2 1.0% benzoic acid mixed to the food. Each group consisted in 40 animals (20 males and 20 females). The N content of the food was controlled

every 6 weeks by the Kjeldahl method.

During the main growth period e.i. in the first 8 weeks, weight was controlled weekly, later every 4 weeks. To test the sexualmaturity and its possible modification by the benzoic acid we paired a male and a female at the age of 11-12 weeks. The pairs stayed together for 14 days. If this "pairing" remained without success, it was repeated after 8 weeks to test the appearance of a retarded sexual maturity or a complete sterility. In the 48th. week of the experiment, another mating (old age pairing) took place to find out if the onset of menopause was normal. We recorded the size of litters on the first and second day as well as the number of reared offspring on the 21 st day.

We also carried out a feeding experiment with a supplement of 5% Na benzoate to the Latz Standard Diet.

Table 1 - Weight gain in gr. Means and standard deviation of means

	Kontrollen		0,5% Benzoesäure		1% Benzoesäure	
	♂	♀	♂	♀	♂	♀
I Generation						
in 4 Wochen	19 ± 5,0	67 ± 1,1	69 ± 2,7	64 ± 2,4	75 ± 1,4	61 ± 2,2
in 8 Wochen	159 ± 6,6	124 [illegible]	160 ± 5,6	135 [illegible]	147 ± 2,8	135 ± 2,4
in 12 Wochen	[illegible]		221 ± 5,6		[illegible] ± 6,0	
II Generation						
in 4 Wochen	69 ± 2,5	[illegible]	[illegible]	60 ± 6,7	73 ± 3,3	70 ± 2,4
in 8 Wochen	167 ± [illegible]	[illegible] ± 5,6	161 ± 2,6	131 ± 3,5	158 ± 4,6	125 ± 3,3
in 12 Wochen	245 ± 5,5		238 ± 5,5		251 ± 5,6	
III Generation						
in 4 Wochen	73 ± 3,0	69 ± 5,5	68 ± 3,8	71 ± 2,4	72 ± 3,5	67 ± 3,7
in 8 Wochen	157 ± 4,5	134 ± 4,1	158 ± 5,5	137 ± 3,9	168 ± 2,0	136 ± 4,5
in 12 Wochen	240 ± 4,6		236 ± 5,8		233 ± 3,8	
IV Generation						
in 4 Wochen	93 ± 1,7	76 ± 4,8	84 ± 2,9	70 ± 3,1	73 ± 5,3	72 ± 3,5
in 8 Wochen	189 ± 5,5	137 ± 4,3	168 ± 3,7	143 ± 2,4	159 ± 6,2	134 ± 4,5
in 12 Wochen	255 ± 4,6		245 ± 4,4		240 ± 4,4	

Table 2

Tab. 2: Protein-Efficiency (Gewichtszunahme je g Nahrungseiweiß)

Generation	Kontrollen		0,5% Benzoesäure		1% Benzoesäure	
	♂	♀	♂	♀	♂	♀
I in 2 Wochen	1,28	1,30	1,37	1,28	1,21	1,30
II in 2 Wochen	1,24	1,24	1,34	1,31	1,46	1,37
III in 2 Wochen	1,66	1,28	1,26	1,33	1,42	1,52
IV in 2 Wochen	1,40	1,26	1,44	1,37	1,37	1,46
I in 4 Wochen	1,27	1,22	1,14	1,08	1,19	1,22
II in 4 Wochen	1,14	1,11	1,19	1,10	1,26	1,20
III in 4 Wochen	1,21	1,06	1,14	1,11	1,27	1,18
IV in 4 Wochen	1,47	1,33	1,41	1,20	1,38	1,37
I in 6 Wochen	1,18	1,10	1,12	1,05	1,07	1,10
II in 6 Wochen	1,10	1,01	1,17	1,05	1,22	1,04
III in 6 Wochen	1,20	1,04	1,08	1,03	1,34	1,09
IV in 6 Wochen	1,30	1,16	1,33	1,17	1,28	1,18
I in 8 Wochen	1,12	0,92	1,00	0,91	0,94	0,92
II in 8 Wochen	1,06	0,90	1,05	0,93	1,10	0,93
III in 8 Wochen	1,04	0,94	1,02	0,92	1,14	0,97
IV in 8 Wochen	1,20	0,99	1,12	0,98	1,12	0,92

Table 3

	% Benzoesäure im Futter		
	0	0,5	1,0
Gesamtzahl der eingesetzten Weibchen	80	78	80
Sterile Weibchen in %	14	10	4
Weibchen mit verzögerter Geschlechtsreife	7,5	17	9
Wurfstärke	9,0 ± 0,35	9,5 ± 0,26	9,6 ± 0,29
Gesamtzahl der geworfenen Jungen	625	688	741
Aufgezogene Jungen in % der Zahl der geworfenen	74	66	63
Alterssparung (2 Generat.)			
Zahl der eingesetzten Weibchen	37	38	38
Wurfstärke	6,9	7,7	7,5
Gesamtzahl der geworfenen Jungen	173	159	171
Aufgezogene Jungen in % der Zahl der geworfenen	72	61	73

RESULTS

Experiments with 5% benzoate in the food

The feeding experiment with 28 young rats (starting weight :62-70 gr) resulted in the death of 19 of the animals with 5% Na benzoate in the food within 14 days. The rest perished in the third week of the experiment. The food intake of the animalswith 39 gr. average in the week was clearly below that of the controls which consume 59 gr. All animals surviving for more than 2 weeks suffered from severe diarrhea. The autopsy of 6 rats which died in the third week only showed hemorrhages in the intestines, moreover blood crusts were visible on the nose. The urine was free from protein and sugar.

In a further experiment 5 adult rats received a diet containing 5% benzoate. 4 of the animals perished in the first 4-5 weeks. Their body weight had decreased from 221-232gr. to 161 gr.

Experiments with 0.5 and 1.0% benzoic acid in food

The results concerning weight gain and food utilization (calculated as "protein efficiency" i.e. weight gain per gr. of food protein consumed) are shown in Tables 1 and 2. In all 4 generations examined there was no effect of the administration of 0.5 and 1.0% benzoic acid on growth and food utilization.

Data concerning reproduction are summarized in Table 3. Since, apart from certain seasonal differences in reproduction and rearing of the young, there were no differences between the 4 generations observed, we show the complete data combined in Table 3 . The litter size was

always a little smaller in the control animals than in animals fed benzoic acid. The control animals mostly have raised more offspring percentually than those receiving benzoic acid which is due to the smaller size of litter. The data obtained by us show no effect of feeding 0,5 and 1.0% benzoic acid on the reproduction and the capacity of raising offspring.

Table 4 - Results on life duration of the animals

	% Benzoesäure im Futter		
	0	0,5	1,0
Zahl der Tiere	78	79	80
Durchschnittl. Lebensdauer Tage	785 ± 20	899 ± 28	827 ± 25
Zahl der Tiere mit einer Lebensdauer von			
unter 600 Tagen	13	8	15
601—800 Tagen	23	18	11
801—1000 Tagen	32	27	42
über 1000 Tagen	10	26	14

Results on life duration of the animals of the first 2 experimental generations are summarized in Table 4. The animals of the third generation were killed after raising their first litter, after the 16, experimental week and examined histologically. The animals of the fourth generation likewise were only observed up to the rearing of the first litter and were then eliminated from the experiment. Since there were no significant differences in lenght of life between the first 2 generations they were both evaluated together in Table 4. The animals receiving 0.5% benzoic acid in their food show a statistically significant prolongation of average lenght of life compared with the controls. On the other hand the figures are no longer statistically significant in the group with 1.0% benzoic acid.

A subdivision of the lenght of life reached in the 4 groups (short-lived animals up to 600 days, one group living 601 to 800 days, one living 801 to 1000 days, and longlived animals with a life time of more than 1000 days) clearly shows the shift in the numbers of animals from short-lived to long-lived in those fed 0.5% benzoic acid. The reason for the life prolonging effect of moderate benzoic acid doses is unexplained. We assume that it is an effect which is analogous to the well known favorable effect of ingestion of chlore-tetra-cycline (aureomycine) and other antibiotics in animal nutrition. The life prolonging effect of the ingestion of benzoic acid would therefore be attributable mainly to an effect on intestinal flora and suppression of undesirable toxine formation. The oldest animal in our experimental series reached an age of 1346 days, it had 1.0% benzoic acid in the food and consumed during its life time altogether over 200 gr. benzoic acid.

The animals of the third group were killed by a blow on the neck after 16 weeks for histological studies. The organ weights found there are reproduced in Table 5. The high liver weights and their larger scatter in the females are due to the weight increase of this organ during suckling of the young and to the varying litter size and rearing of the offspring.

The histological examination of the tissues was generously carried out by Prof. DOMAGK (Elberfeld) whom we sincerely thank for his labors. It showed no evidence for a toxic effect of benzoic acid.

A systematic examination of causes of death was not carried out by us

for external reasons. According to the studies of HOSINO (6) benzoic acid is not cancerogenic even after parenteral application.

DISCUSSION

Our experiments have shown that 5% benzoate in the food is with certainty toxic, but that 1% benzoic acid does not manifest any toxic effects even after observation extending over the whole life time and administration to 4 generations.

Benzoic acid is conjugated in the organism with glycine to give hippuric acid and is excreted in urine in this form. In addition to a smaller extent there is conjugation with glucuronic acid but, even after extremely high benzoic acid doses, excretion of the glucuronide accounts at most for 10% of the total benzoic acid excretion. The maximal rate of hippuric acid formation has been determined for the rat as 36 μmoles per hour per 100 gr. body weight provided the necessary amount of glycine can be made available (SIMKIN). In humans it is 122 to 155 μmoles per minute i.e. appr. 1 gr. benzoic acid per hour (SCHACHTER). For the detoxication of benzoic acid only a small fraction, about 1/3 of alimentary glycine is utilized, even after the ingestion of very large quantities of free glycine, the largest proportion of the glycine utilized for detoxication is newly synthetized in metabolism. This explains why the protein content of the food is of the highest importance for the tolerance of administered benzoic acid. In our experiment protein supply was 22.5%, hence within the optimal limits for rats.

1% benzoic acid in food corresponds to a daily intake of app. 150 mgr.

or 0.45 μmoles per 100 gr. body weight. For the detoxication of this quantity of benzoic acid 0.45 moles of glycine per 100 gr. body weight is required. The maximum rate of detoxication was found to be 0.864 μmoles per day per 100 gr. body weight. therefore not quite double that required for the detoxication of 1% benzoic acid in food. This shows that the conditions for the detoxication of benzoic acid at doses exceeding 1% in the food are beginning to become critical. 1% benzoic acid in food administered chronically must therefore be regarded as close to the upper limit of the non-toxic range. Reports contained in the literature on feeding experiments (see the review of LANG (8)) confirm this assumption.

Table 5 - Organ weights of third generation animals in % of body weight

		Kontrollen	0,5 % Benzoesäure	
	Körpergewicht in g	[illegible]	[illegible]	[illegible]
Brain	Gehirn	0,57 ± 0,11	0,58 ± 0,01	[illegible]
Heart	Herz	0,54 ± 0,02	[illegible]	[illegible]
Liver	Leber	3,49 ± 0,1	3,54 ± 0,1	[illegible]
Spleen	Milz	0,21 ± 0,00	[illegible]	0,01
Kidney	Nieren	0,72 ± 0,02	[illegible]	± 0,02
Testes	Testes	[illegible]	[illegible]	[illegible]

Experiments with radioactive benzoic acid have confirmed the old finding that the substance is not accumulated in the organism, but is quantitatively excreted within a very short time, provided the limiting dose mentioned above is not exceeded. (BERNHARD (1) and LANG (9)). The discovered pharmacological effects of benzoic acid observed at

the lowest concentration (dilatation of renal vasculature, impairment of prostigmine effect on muscle (BUBNOFF et al (2)) ,and which can only be obtained by intra-arterial injection required benzoic acid concentration in blood of about 10^{-3} M. According to the studies of LANG with radio active benzoic acid, these concentrations are not reached with doses of 200 mgr. benzoic acid per kg. body weight. 24 hours after such doses the benzoic acid concentration in the blood is about 10^{-8} M and in the liver 10^{-6} M.

On the basis of the probably too unfavorable supposition that 1% benzoic acid in food is the upper limit which is still tolerable for the rat during chronic administration (corresponding to a daily intake of about 150 mgr. per animal, a daily dosis of 34.6 gr. benzoic acid would be calculated for a 70 kg, man - relative to the respective body weight.

The maximal acceptable daily dosis of 0.6 gr. benzoic acid proposed by the Commission on Preservatives of the German Society for Research and accepted by the legislation therefore offers a sufficient margin of safety compared with the maximal dose determined experimentally in chronic feeding experiments and calculated from metabolic data and therefore appears to be well founded.

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The Journal of Biochemistry, Vol. 41, No. 4, 1954

INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

III. EFFECTS OF SODIUM SALICYLATE AND BENZOATE UPON THE REACTION OF CHOLEGLOBIN FORMATION

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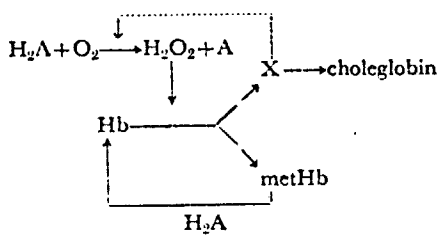
(Received for publication, April 17, 1954)

For the reaction process of choleglobin formation in the system of hemoglobin—ascorbic acid—oxygen, a reaction scheme has been presented (*1*) on the basis of our experimental evidences as follows:



where H_2A is ascorbic acid.

In the above scheme, an intermediate compound x was introduced as a presumption. The intermediate x was postulated to be a hemoglobin derivative which arises from native hemoglobin through a slight grade of denaturation in its globin part. The intermediate may be much more active as an oxidative catalyst than the native hemoglobin, thus being responsible for the chain reaction nature of the process of choleglobin formation.

On the other hand, Tsushima (*2*) in our laboratory reported recently on his detailed studies regarding a slight and reversible denaturation of various hemoglobin derivatives, such as reduced Hb, metHb and HbO_2, which was caused by the action of sodium salicylate and benzoate, respectively. The denaturation in these cases were proved to be completely reversible in the meaning of Holden's "perturbation". It is of special interest in Tsushima's paper, that the autoxidation of HbO_2 was also proved to be accelerated significantly by those

"perturbators". Holden (*3*) reported in 1947, that a series of reagents being able to perturb hemoglobin under suitable conditions, promoted the formation of green pigment; this was observed by repeated desoxygenation and reoxygenation of horse HbO_2 by $Na_2S_2O_4$ and by atmospheric oxygen alternatively, or by the addition of H_2O_2 in the presence of $Na_2S_2O_4$. In Holden's paper, a suggestful quotation was made as follows: "Since a lower concentration of a perturbator than is required for a visible degree of perturbation is adequate for the promotion of green pigment formation, one is tempted to assume an intermediate step whereby a little of the hemoglobin is modified in such a way that the molecule oxygen neither unites with iron atom nor so reacts as to denature the protein, but takes a third course".

His assumption, however, has not heretofore been susceptible of experimental verification, since neither the nature of the "perturbation" of hemoglobin molecule nor its relation to the process of green pigment formation could be ascertained. His observations were only qualitative.

The present work was undertaken with the aim of determining the underlying relationship between the structural and functional modifications of hemoglobin molecule induced by salicylate and benzoate, respectively, with special references to their influences upon the reaction rate of choleglobin formation.

EXPERIMENTAL METHODS

Crystalline equine and bovine HbO_2 for our experimental purpose were prepared following the method of Heidelberger (*4*) improved in some points by Suzuki *et al.* (*5*). The samples contained neither catalase nor metHb in their fresh state. Crystalline HbO_2 was dissolved in redistilled water as a stock solution. This was diluted to 1.5×10^{-4} *M* of heme concentration in each time at use.

Procedure—Into small beakers are placed 2.5 ml. of HbO_2 solutions, 1.0 ml. of sodium salicylate or benzoate solution in various concentrations were added and were incubated at 37° for 15 minutes. Then, 0.5 ml. of 0.8 *M* ascorbic acid neutralized with equivalent NaOH was added into each of the beakers and were kept standing at 37° so as to be reacted by atmospheric oxygen. The reaction was stopped by the addition of NaOH at each interval of time. The reaction solution was then reduced by addition of a small amount of powdered $Na_2S_2O_4$ and the choleglobin in the solution was measured spectrophotometrically. The readings were made by a Hitachi photoelectric spectrophotometer. The amount of choleheme formed was calculated following the procedure described in our previous paper (*1*) according to the formulations presented in the same place. Oxygen uptake mainly for the ascorbic acid oxidation was measured manometrically by Warburg's technique.

RESULTS

Experiments with Sodium Salicylate—The reaction rate of choleglobin formation, as well as the final values of cholcheme formed, increased with increasing concentration of salicylate unless it exceeded over a certain limit. When, however, the salicylate concentration exceeded this limit, the reaction rate decreased and the final amount of cholcheme formed remained far below its maximum value (Fig. 1). The same was

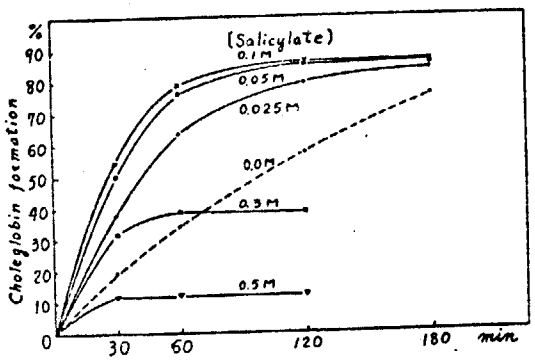


FIG. 1. Choleglobin formation in the presence of sodium salicylate. 2.5 ml. of horse HbO_2 (1.5×10^{-4} *M* as the heme concentration), 1.0 ml. of salicylate of varied concentration, 0.5 ml. of 8×10^{-2} *M* ascorbic acid. T=37°

The numbers designated in the figure represent the final concentrations of salicylate in the reaction mixture.

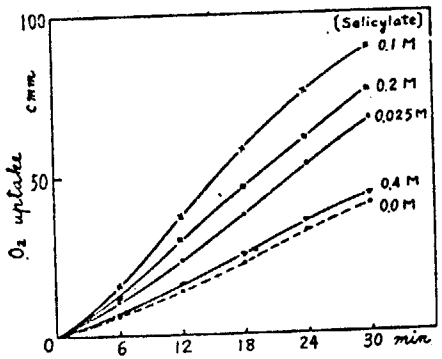
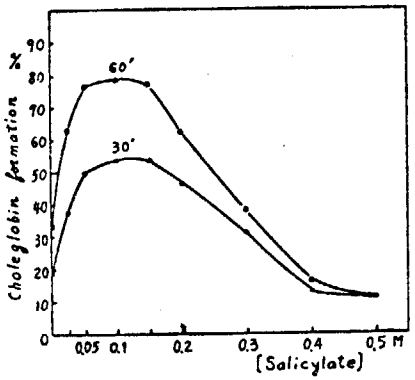


FIG. 2. O_2 uptake in the presence of sodium salicylate. Composition of the reaction mixture and temperature were the same as described in Fig. 1.

found in the O_2 uptake in the same reaction series (Fig. 2). The effect of salicylate varying in concentrations upon the present reaction can be expressed by the changes in the reaction rate in percentages to that of the control experiments without salicylate. Both the rate of choleglobin formation and that of O_2 uptake reached the maximum value at the concentrations of salicylate within 0.05 to 0.15 *M*. Within this range of concentrations the rates were found to be constant indicating a distinct plateau in each of the curves in Figs. 3 and 4. The same was observed also in the experiment with bovine HbO_2 but rather within wider concentration range of 0.05 to 2.0 *M*.



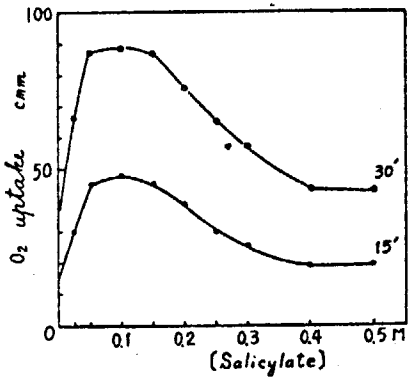


FIG. 3. Effect of sodium salicylate added in various concentrations upon the reaction rate of choleglobin formation (derived from Fig. 1). Comparison was made with the values of choleglobin formed during 30 and 60 minutes of the reaction.

FIG. 4. Effect of sodium salicylate added in various concentrations upon the rate of O_2 consumption (derived from Fig. 2).

Comparison was made with the values of O_2 uptake during 15 and 30 minutes of the reaction.

Experiments with Sodium Benzoate—The effect of benzoate upon the reaction of choleglobin formation was proved to be similar to that of salicylate, as shown by the Curves II and II′ in Fig. 5. For the promotion of choleglobin formation, higher concentration was needed of benzoate than of salicylate. The range of benzoate concentrations within which a maximum and a constant promotion of the rate was observable, was found to be much wider than in the case of salicylate. In these cases with benzoate, no appreciable difference in the reaction could be proved between bovine HbO_2 and equine HbO_2.

Effect of the Prolonged Incubation of HbO_2 with the Perturbators—The perturbation of hemoglobin proceeds with time as reported by Tsushima (*2*). Thus, it seems to be of some interest to investigate the effect of prolonged incubation of HbO_2 with salicylate

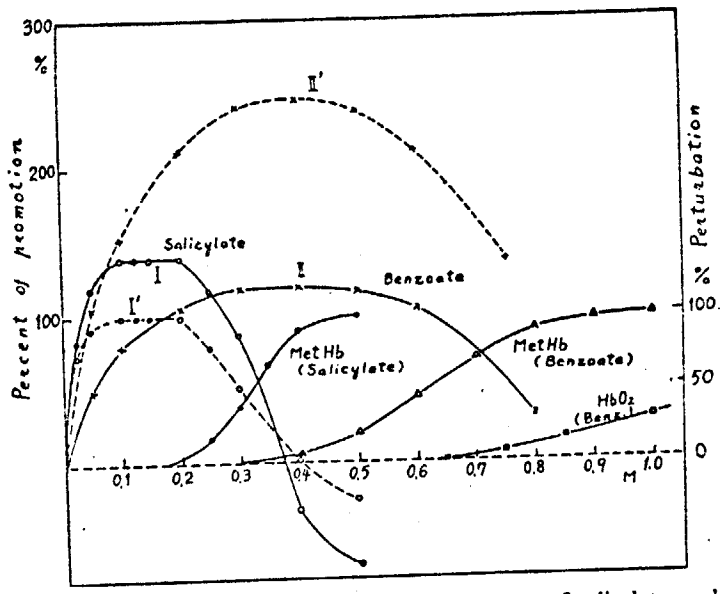


FIG. 5. Percentage representation of the effects of salicylate and benzoate upon the reaction of choleglobin formation. Bovine hemoglobin was used in these series of experiments.

I. Effects of salicylate upon the choleglobin formation.
I'. Effects of salicylate upon the O_2 consumption.
II. Effects of benzoate upon the choleglobin formation.
II'. Effects of benzoate upon the O_2 consumption.

The curves without denotation of numbers represent:

—●—●— Per cent of the visible degree of perturbation of metHb by sodium salicylate.
—△—△— The same by sodium benzoate.
—□—□— Per cent promotion of the autoxidation of HbO_2 by benzoate.

upon the choleglobin formation. For the purpose, HbO_2 was first incubated with salicylate for various lengths of time and thereafter the reaction of choleglobin formation was started by addition of ascorbic acid. With the concentration of 0.125 *M* of salicylate, no appreciable effect of incubation time was recognized upon the choleglobin formation, while in the salicylate concentration of 0.25 *M*, both the rate of the reaction and the final yield of choleglobin decreased according to the length of incubation time (Fig. 6).

Effect of the Temperature of Incubation—The effect of temperature upon the reaction of choleglobin formation in the presence of salicylate or benzoate was very significant

and characteristic. The temperature effect on the reaction rate was much greater in the presence of salicylate or benzoate than without them (Figs. 7 and 8). While at ralatively lower temperatures, the formation of choleglobin increased with increasing concentrations of salicylate or benzoate; at higher temperatures, however, the maximum rate was observed only within a definite concentration range of perturbator. The optimum shifted gradually toward the lower concentrations of perturbators as the temperature became higher. These facts can only be plausibly explained by the concept that the perturbation is a sort of protein denaturation.

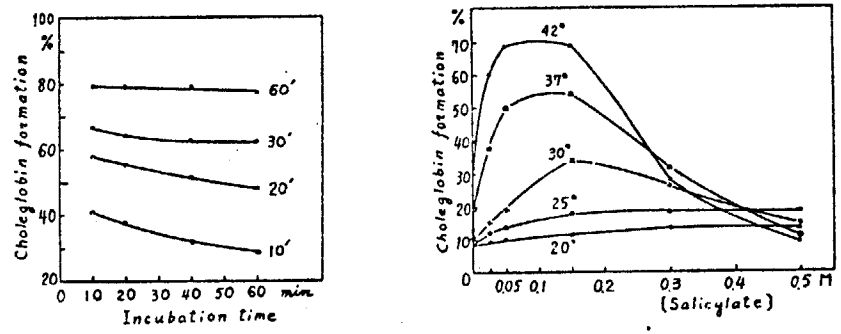


FIG. 6. Effect of the incubation time upon the choleglobin formation. Concentration of salicylate in the reaction mixture was 0.25 *M*. The numbers in the figure represent the time in minutes after the reactions of choleglobin formation started on addition of ascorbic acid.

FIG. 7. Effect of the temperature upon the choleglobin formation in the presence of salicylate in various concentrations.

Comparison was made with the values of choleglobin formed during 30 minutes of the reaction.

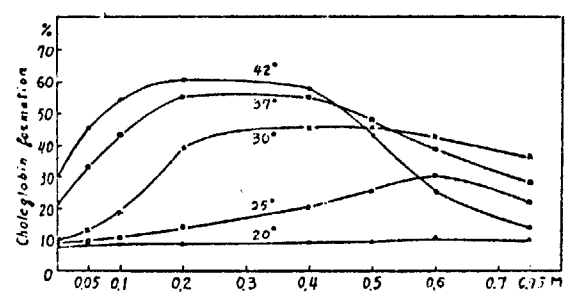
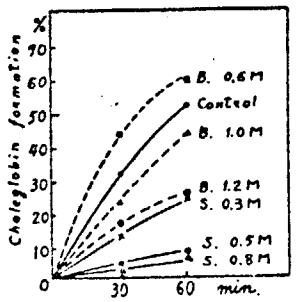


FIG. 8. Effect of the temperature upon the choleglobin formation in the presence of benzoate in various concentrations.

Comparison was made with the values of choleglobin formed during 30 minutes of the reaction.

Reactions Taking Place after the Visible Degree of Perturbation has been in Completion—Bovine methemoglobin was used in this reaction series. The concentrations of salicylate and benzoate were kept highly enough to bring about the visible degree of perturbation in completion within the incubation of 15 minutes. The results are shown in Figs. 9 and 10. In cases with salicylate, the rates of both the choleglobin formation and the O_2 uptake were very small, while in cases with benzoate the reaction proceeded steadily with time, the rate of O_2 uptake being found to be greater than that of control experiment.



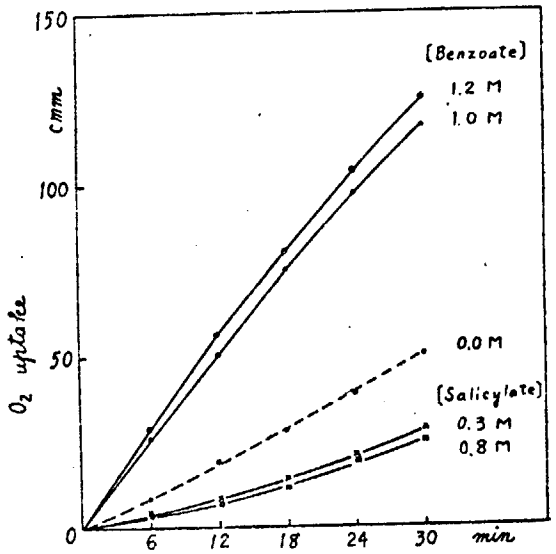


FIG. 9. Choleglobin formation in the presence of sufficiently high concentration of salicylate or benzoate as enough to bring about the perturbation of visible degree of hemoglobin. Bovine metHb was used in this series of experiments. Other reaction conditions were the same as described in Fig. 1.

FIG. 10. O_2 uptake in the same reaction conditions as described in Fig. 9.

DISCUSSION

From the results presented here, it is evident that salicylate and benzoate promote the oxidative activity of hemoglobin as well as the decomposition of the latter into choleglobin in the presence of ascorbic acid as the substrate. A mild denaturation of protein part affected by

salicylate or benzoate may be thus responsible for the promotion of oxidative activity of hemoglobin molecule. And when the protein part is affected one step further, an inactivation takes place instead. The same conclusion can be drawn from the results of experiments with longer incubation at various reaction temperatures. This sort of stepwise modification by these denaturants according to their concentration gradient could also be observed by Shukuya (*6*) and Tsushima (*2*). In Fig. 5, the results of Tsushima (*2*) indicating the effective concentrations of salicylate and benzoate for the perturbation of methemoglobin and for the promotion of HbO_2 autoxidation were additionally given beside our results. As seen from the figure, the concentrations of salicylate and benzoate favoring the promotion of choleglobin formation were found to be lower than that required for the spectral changes of methemoglobin into hemichrome and were far lower than those demanded for the promotion of HbO_2 autoxidation. These facts seem to be suggesting clearly the possibility of stepwise processes of the structural modifications taking place in hemoglobin molecule. In conclusion, some secondary stable state of hemoglobin molecule which is highly active as an oxidative catalyst might be formed as an intermediate of hemoglobin denaturation, and once the denaturation proceeded so profoundly as to the visible degree of perturbation of hemoglobin, the oxidative activity of the hemoglobin would be lost again. It may not be surprising in these cases if the unfolded protein molecule exhibits their functional modification not by loss but by alteration of biological activity, or of catalytic activity when we speak of heme-proteins. And, as suggested by our presentation, there can be many of the criteria for the demonstration of structural modifications in protein molecule, and while one can evidence a structural change by some of these criteria, but sometimes may not by others.

It is doubtful whether salicylate affects the hemoglobin molecule as similarly as benzoate. As shown in Fig. 5, an adequate concentration of salicylate for the perturbation was lower than that of benzoate, and furthermore the optimum concentration range of salicylate was found to be narrower than that of benzoate. While, moreover, salicylate is effective to promote the choleglobin formation more strongly than the oxidative activity of hemoglobin, benzoate is effective in reverse relation. The results shown in Figs. 9 and 10 also present a question whether the effects of salicylate and benzoate are similar. Perturbation of hemoglobin by benzoate may qualitatively be of milder grade than by

salicylate.

In our previous paper, it was postulated that in the process of choleglobin formation, an intermediate compound x may be formed from hemoglobin, seemingly the protein part of the latter being mildly modified in some sort of denaturation and that this x may be playing an active role in the process of choleglobin formation. This postulation receives further verification by the present experiments.

Many investigations have been hitherto made for the elucidation of the possible relationship between the molecular constitution of hemoglobin and its function. Beside the eminent works of Pauling (*7*) and of Wyman (*8*) on the well known Bohr effect, recent investigations of Stary (*9*) and of Riggs (*10*) can be pointed out in this respect. Stary and Tekman indicated that the protein part of hemoglobin molecule might be stabilized by the attachment of CO to hemoglobin more profoundly than by that of O_2. On the other hand, Riggs has drawn attention to the fact that SH-groups in hemoglobin molecule seem to be closely connected with the mechanism of heme-heme interaction, and the fact seems to be of some importance for the elucidation of our present investigation. Also Laidler's thermodynamic studies (*11*, *12*) on the reaction mechanism of urease or pepsin seem to be suggestive to our present problem.

SUMMARY

Studies were made on the changes of catalytic activities of hemoglobin following the structural modification of the latter induced by sodium salicylate or benzoate with special reference to their influences upon the process of choleglobin formation in the hemoglobin—ascorbic acid—O_2 system. The results are summarized as follows:

1. Reaction rate of choleglobin formation, as well as of O_2 uptake, increased with increasing concentrations of salicylate or benzoate added. But, when the concentration of the perturbators increased over a definite limit, the rate decreased again.

2. The perturbation of hemoglobin molecule seemed to proceed stepwise following the increase of salicylate or benzoate concentrations. There may most probably be a secondary stable state of hemoglobin molecule.

3. The concentration of salicylate or benzoate favorable to promote the choleglobin formation was proved to be far lower than that either to bring about the spectroscopically demonstrable grade of perturbation of hemoglobin molecule or to promote the rate of HbO_2 autoxidation.

4. The temperature effect on the reaction of choleglobin formation under the presence of perturbators was much greater than that without perturbators.

5. From all of the results of the present experiments, it may be postulated that the above mentioned effects of benzoate and salicylate are due to the protein denaturation of a milder grade caused by them. Our previous assumption of an active intermediate compound x possibly produced in the process of choleglobin formation from hemoglobin may be accepted to be justifiable on the basis of our present experimental evidences.

The authors wish to thank Prof. K. Kaziro for his continous guidance and encouragement during the course of the present studies.

This work was aided by a Grant from the Scientific Research Fund of the Ministry of Education (K. Kaziro).

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Studies on Metabolism and Identification of the Causative Agent in Aspirin Teratogenesis in Rats[1,2]

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ABSTRACT Pregnant Wistar rats treated orally at day 9, 10, or 11 with 250, 500, 750, or 1000 mg/kg aspirin showed a teratogenic dose-response relation at each treatment time, but a decreased overall embryonic susceptibility as treatment was applied later in gestation. The types of abnormalities produced generally correlated with the state of development at the time of treatment. Treatment of animals at day 9 with 510 mg/kg benzoic acid and 2 hours later with 250 or 500 mg/kg aspirin caused a significant increase in percentage of malformations above effects observed after 250 or 500 mg/kg aspirin alone. Determination of free salicylate in embryos and maternal serum after treatment at day 11 with sodium salicylate with or without benzoic acid pretreatment indicated that benzoic acid significantly increased the concentration of salicylate in both embryos and serum 6 and 12 hours after salicylate treatment and throughout the observed period. Thin-layer chromatography of tissues from these animals revealed only salicylic acid in both embryos and maternal serum, thus implicating salicylic acid as the causative agent in aspirin teratogenesis.

Aspirin and other salicylates have been recognized as teratogens in rats since the first report by Warkany and Takacs ('59), and subsequent studies have shown that salicylates are teratogenic in mice (Larsson et al., '63; Trasler, '65) and monkeys (Wilson, '70) but not rabbits (Earley and Hayden, '64; McColl et al., '67). The abnormalities included central nervous system defects (craniorachischisis), severe skeletal defects, gastroschisis, and facial abnormalities (Warkany and Takacs, '59; Trasler, '65; McColl et al., '67; Smith and Monie, '69).

Several investigations on possible mechanisms of salicylate teratogenic action have been reported. Larsson and Boström ('65) and Larsson et al. ('67) studied the effects of salicylates on acid mucopolysaccharide synthesis in mouse embryos. Goldman and Yakovac ('63, '64a,b, '65) reported on maternal stress and its relation to salicylate teratogenicity but did not present conclusive data to support their hypothesis. Recently, Janakidevi and Smith ('69) reported the effects of salicylates on RNA polymerase activity in 13 and 16-day rat fetuses, indicating a decrease in activity and thus an inhibition of RNA synthesis.

The primary aim of the study reported here was to determine the possible site(s) of teratogenic action of aspirin and to identify the causative agent, whether aspirin, salicylic acid, or some other of the possible metabolites (fig. 1).

MATERIALS AND METHODS

Female Wistar rats (Albino Farms) were placed overnight in cages with males and examined for sperm the next morning by vaginal lavage. Nine o'clock on the morning of finding sperm was considered time 0 of pregnancy. Females were housed after mating in groups of two in wire cages and allowed commercial rat chow

[1] Supported by NIH grant HD02792 to J. G. Wilson.

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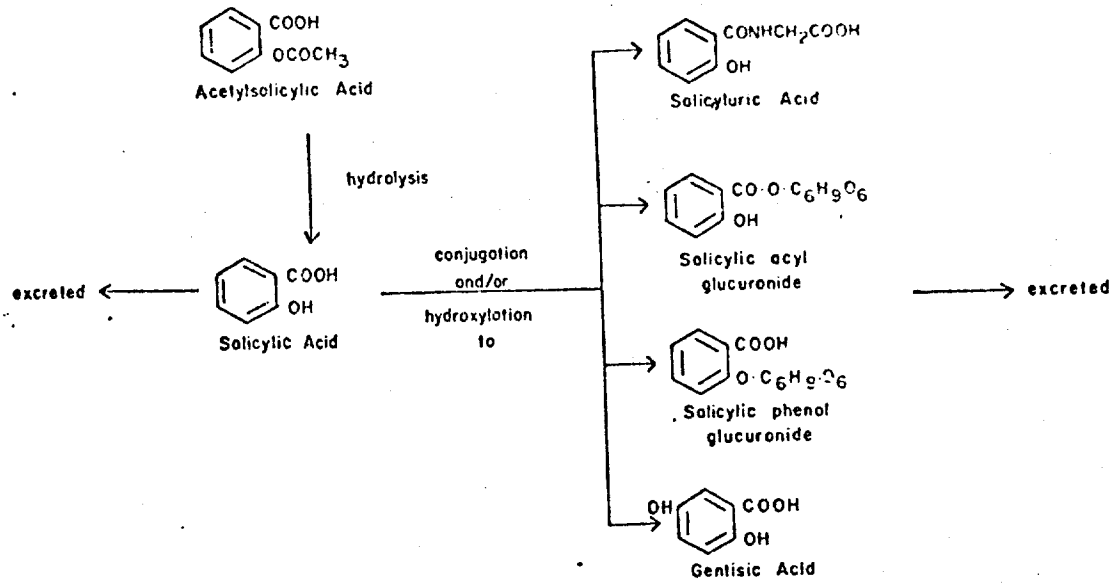

Fig. 1 Pathways of biotransformation and excretion of aspirin in man and rats.

and water ad libitum. Females weighing 200 to 250 g were treated by gavage at day 9, 10, or 11 of gestation (i.e., at approximately 216, 240, or 264 hours after time 0) with 250, 500, 750 or 1000 mg/kg aspirin[4] suspended in 0.2% carboxymethylcellulose (CMC). Each animal received 5 ml drug or CMC per kg body weight. (Ether was not used to facilitate treatment by gavage since a large number of cases of hydronephrosis and hydroureter were found in CMC-treated control females anesthetized with ether.) Hyperventilation was noted for a few hours after treatment in females given salicylates, and in some animals a weight loss of 2-10 g was recorded within 2 or 3 days after treatment, but by day 20 of gestation the weights of these animals were comparable to controls. The animals were killed at day 20 of gestation and the uterus observed in situ for implantation and resorption sites. Live fetuses were removed, examined for gross malformations, and weighed. Two-thirds were fixed in Bouin's solution and the remainder in 95% ethanol. Fetuses fixed in Bouin's were examined for internal malformations by razor-blade sectioning, while fetuses in alcohol were cleared in 2% potassium hydroxide, stained with alizarin red S, and after further clearing stored in pure glycerin (Wilson, '65). Skeletal examination was then carried out under low magnification.

To determine the teratogenic effect of increased and prolonged maternal serum concentration of salicylic acid other pregnant animals were treated orally at day 9 of gestation with 510 mg/kg benzoic acid in CMC followed 2 hours later by 250 or 500 mg/kg aspirin orally. (The large dose of benzoic acid was arbitrarily chosen to assure prolonged, total depletion of the glycine pool.) Animals were killed at day 20 and fetuses examined for malformations as outlined above.

Determinations of free salicylate in maternal serum and embryos were carried out at day 11 of gestation at which time embryos can be removed and analyzed individually. For this purpose pregnant animals were treated orally with 510 mg/kg benozoic acid and 2 hours later orally with 500 mg/kg sodium salicylate. Other animals were treated with 500 mg/kg sodium salicylate alone. Sodium salicylate was used because (1) it is soluble in water, making it easier to administer

[4] Acetylsalicylic acid (powdered) was purchased from Mallinckrodt Chemical Works, New York. Crystalline sodium salicylate was purchased from Squibb and Sons, New York. Gentisic acid (2,5 dihydroxybenzoic acid) was purchased from Eastman Kodak Co., Rochester, New York.

and insuring a more accurate dosage; (2) its teratogenic effects are identical or very similar to those of aspirin (Warkany and Takacs, '59; Larsson and Eriksson, '66); and (3) only salicylic acid is found in the circulation, owing to the rapid hydrolysis of aspirin to salicylic acid after absorption from the gut.

Treated animals were placed in metabolism cages and urine was collected during the time between salicylate treatment and killing, diluted 1:4 with 0.01 N HCl, and stored at 0–4° C. At 3, 6, 12, or 24 hours after sodium salicylate treatment 3–4 ml of blood were taken by heart puncture and the animals were then killed. The uterus was opened and embryos were dissected from surrounding membranes, weighed on a Cahn electrobalance, placed in 1 ml of 0.01 N HCl, and all samples stored at 0–4°C. Salicylate concentration was determined by the method of Chirigos and Udenfriend ('59), which was modified as follows: (1) embryos were sonicated (Bronwill Biosonic III) in 1 ml 0.01 N HCl (centrifugation was omitted, owing to the small amount of cellular material present at this developmental stage), and (2) 1,2-dichloroethane (spectroquality) was saturated with distilled water and used for the extraction of salicylate.

Aspirin and its transformation products were identified by thin-layer chromatography in embryos, maternal sera, and urine obtained from animals used for the analytical determinations. Four or five embryos from each litter were pooled and homogenized in 0.5 ml methanol and 0.5 ml water. Aliquots of these specimens plus reference compounds were chromatographed on Eastman chromagram sheets coated with silica gel using a benzene: ether:acetic acid:methanol (120:60:18:1, v/v) solvent system (Cummings et al., '66). Following preliminary ultraviolet examination, sheets were sprayed with ferric chloride to visualize separation of salicylic acid and its phenolic conjugates.

In addition to the studies on salicylic acid two other transformation products of aspirin — salicyluric acid and gentisic acid — were tested to determine their possible teratogenic effects. Salicyluric acid[5] suspended in 0.2% CMC was given orally in dosages of 400 mg/kg at day 9 of gestation. The more lipid-soluble synthetic ethyl ester of salicyluric acid[5] $CONHCH_2CO_2C_2H_5$ OH was also administered at day 9 in dosages of 400 mg/kg suspended in CMC. Other females were treated at day 9 with 750 mg/kg gentisic acid[4] in CMC. Females were killed at day 20 and fetuses removed and examined for malformations as described earlier.

RESULTS

Teratogenic effect of aspirin at days 9, 10, 11 of gestation

Table 1 shows the teratogenic potency of aspirin treatment at days 9, 10, and 11 of gestation. A dose-response relation was indicated by an increase in both mortality and malformation rates with increasing dosage at each of the three treatment times. However, as development advanced from day 9 to 11, larger dosages

[5] Salicyluric acid and the ethyl ester of this compound were synthesized by Dr. H. J. Schumacher and Dr. J. Terapane of Kettering Laboratories, Cincinnati, Ohio.

TABLE 1

Teratogenicity and embryolethality of aspirin at different dosages and gestational ages in rats

Day of gestation	Dosage	No. of females	Total implants	% dead or resorbed [1]	% abnormal survivors [1]
	mg/kg				
9	250	5	60	3	21
9	500	7	89	34	76
10	500	10	128	18	82
10	625	10	121	34	80
11	500	10	118	9	23
11	750	14	145	23	81
11	1000	10	112	59	100
11	Vehicle	6	63	10	4

[1] In this colony, untreated controls show a mortality rate of 4% and malformation rate of 1%.

were needed to produce similar quantitative effects; e.g., 500 mg/kg at day 9 caused 34% dead or resorbed and 76% malformed fetuses, while 750 mg/kg was needed at day 11 to approximate this effect (23% dead or resorbed and 81% malformed).

The types of malformations produced after aspirin treatment at the different times are listed in table 2. A relation existed between the malformations observed and developmental events occurring at the time of treatment. For example, at day 9 the three germ layers were present and only the primitive node and earliest indications of the neural plate were formed. The primary defects after treatment at day 9 involved the central nervous system (craniorachischisis), skeleton, and ventral body wall (gastrochisis). At day 10 of gestation the heart was beginning to form and the neural tube was closing. Abnormalities of the heart (marked levorotation of the cardiac apex resembling levocardia, fig. 2) and brain (hydrocephalus) predominated after treatment at day 10. Embryos at day 11 had the beginnings of forelimb buds, but hindlimb buds had not appeared, and the mesonephric kidney was not visible. Most of the abnormalities produced by treatment at day 11 were of the hindlimb (clubfoot and polydactyly, fig. 3), tail, and kidney. The hindlimb abnormality primarily involved the postaxial border (fibular hemimelia) and in severe cases consisted of complete absence of the bones of the hindlimb (fig. 4). The kidney abnormalities included supernumerary and fused supernumerary kidneys (fig. 5) and may have resulted from interference with earliest differentiation of the ureteric bud, which was first visible at day 12.

Many of the defects produced at each treatment time involved organ systems the primordia of which were not present at the time of treatment (e.g., gastrochisis at day 9, or hindlimb and kidney defects at day 11), and it is likely that the cellular precursors of these systems were affected at the biochemical level, resulting in abnormal early differentiation. Some defects occurred in apparently identical form after treatment at different times, e.g., hydrocephalus and diaphragmatic hernias at day 9 or 10, and cleft palate at day 10 or 11. These situations can perhaps be attributed to relatively slow differentiation through certain teratogenically susceptible stages of organogenesis.

Identification of the causative agent in aspirin teratogenesis

Levy et al. ('69) reported that benzoic acid increased the concentration and persistence of salicylic acid in the plasma by competing for available glycine and thus inhibiting formation of salicyluric acid; and Davison and Smith ('61) reported that benzoic acid competes with salicylic acid for plasma protein binding sites. To increase the concentration of salicylic acid a large dose of benzoic acid was given to pregnant rats 2 hours before aspirin treatment. The effects on teratogenicity of aspirin are presented in table 3. It is apparent that such treatment increased the frequency of malformations significantly, e.g., 100% when 250 mg/kg aspirin were used.

TABLE 2

Types of malformations exhibited after aspirin treatment at different gestational ages

Day 9 — 500 mg/kg (76% survivors abnormal)	Day 10 — 625 mg/kg (80% survivors abnormal)	Day 11 — 750 mg/kg (81% survivors abnormal)
Craniorachischisis (31%)	Hydrocephalus (42%)	Clubfoot (62%)
Skeletal dysplasia (29%)	Levorotation of heart (34%)	Polydactyly (41%)
Gastroschisis or umbilical hernia (22%)	Extra 14th ribs (34%)	Abnormal tail (38%)
Diaphragmatic hernia (18%)	Cleft lip, palate (19%)	Fused supernumerary kidney (20%)
Hydrocephalus (11%)	Hydronephrosis (19%)	Supernumerary kidney (18%)
Eye abnormalities (11%)	Meningocele (14%)	Cleft palate (10%)
Clubfoot (9%)	Eye abnormalities (13%)	Ectopic kidney (7%)
Spina bifida (4%)	Diaphragmatic hernia (11%)	Ectopic testis (2%)
Other (13%)	Cleft palate (6%)	Other (4%)
	Other (13%)	

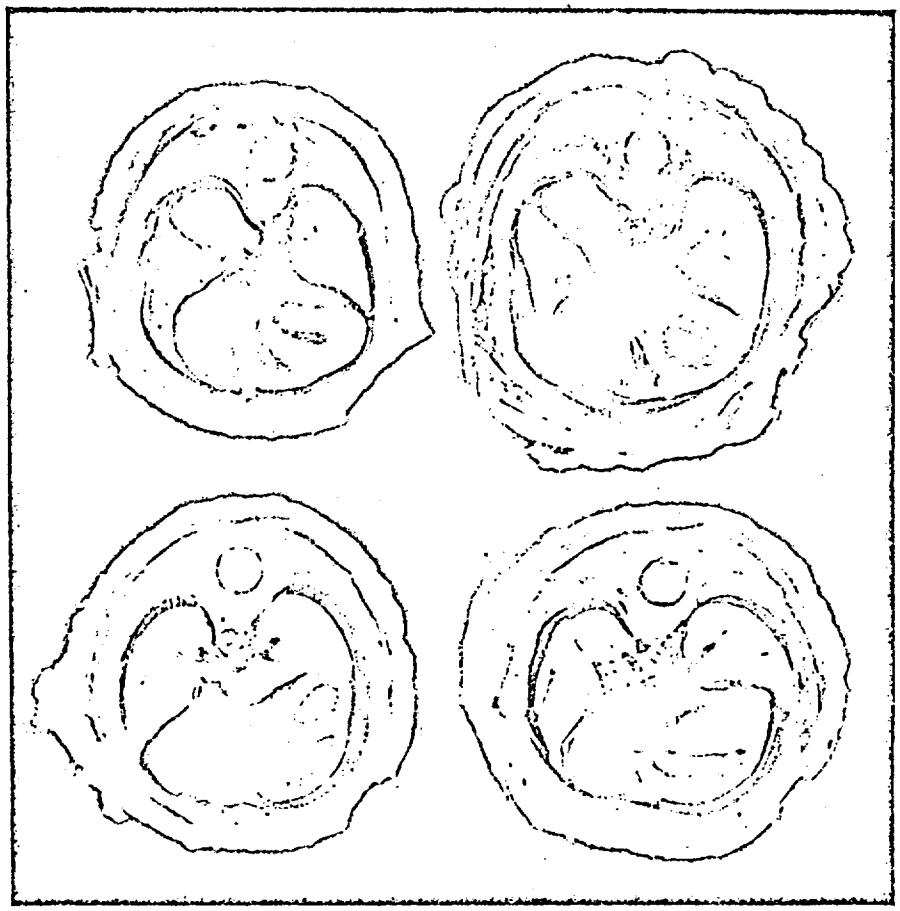

Fig. 2 Sections of rat fetuses with levocardia from maternal animals treated at day 10 Upper right, normal; other sections, various degrees of rotation of heart to the left. × 5.

The types of malformations occurring after treatment with benzoic acid plus aspirin were the same as those produced by aspirin alone at day 9, although some types of abnormalities seen after treatment at day 10, such as levorotation of the heart, were present in these animals as well, and the malformations were in many cases more severe than with aspirin alone. The malformations and resorption rates after benzoic acid alone were comparable to those in control animals.

The concentration of free salicylate in embryos and maternal serum at day 11 is shown in figure 6. An average of 15 embryos from three litters is represented by each point except at 24 hours when only one litter (five embryos) was tested. The highest measured concentration of total salicylate after treatment with sodium salicylate alone occurred at 3 hours, with a sharp decrease thereafter. There was no statistically significant difference between the salicylate concentrations at 3 hours with or without benzoic acid, in serum or embryos, but benzoic acid significantly increased salicylate concentration in both at 6 and 12 hours after salicylate treatment ($P < 0.05$ for serum; $P < 0.02$ for embryos).

The 24-hour salicylate concentration in embryos or serum after treatment with benzoic acid plus sodium salicylate was very similar to the 6- and 12-hour values after sodium salicylate alone, suggesting that enough salicylate may still be present after 24 hours to have a teratogenic effect. This appears to have been the case in the

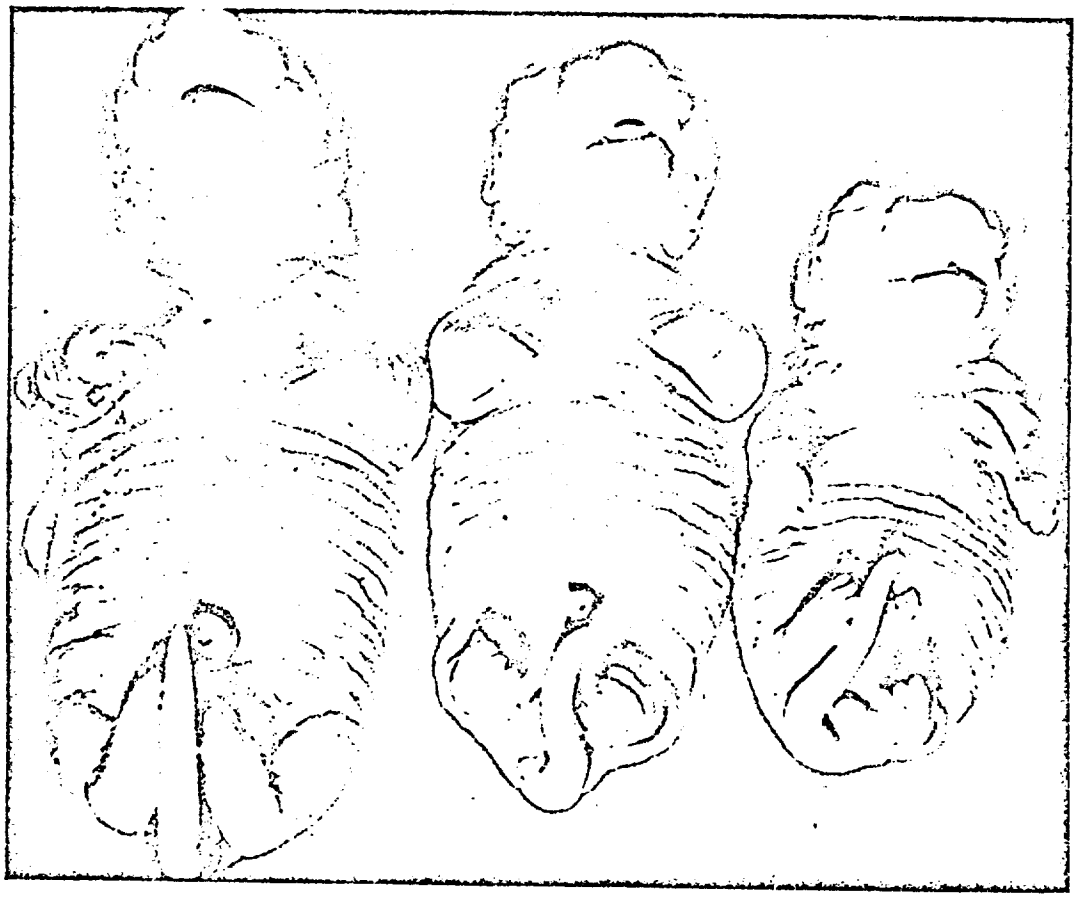

Fig. 3 Left, normal; middle and right, offspring of animals treated at day 11, with club-foot, polydactyly, and abnormal tails. × 2.

animals treated at day 9 with benzoic acid plus aspirin, which exhibited types of malformations usually produced by treatment at day 9 as well as some occurring after treatment at day 10. Thus, benzoic acid greatly increased the time required for total salicylate excretion from the body and thereby increased duration of embryo exposure.

Separation of metabolites by thin-layer chromatography revealed that all of the metabolites of aspirin were present in maternal urine, the predominant one being salicylic acid. Surprisingly, very little salicyluric acid was seen, even in samples from females given sodium salicylate alone, but glucuronides were very evident. In serum and embryos, however, only salicylic acid could be demonstrated with or without benzoic acid pretreatment.

The results of experiments with salicyluric acid, the synthetic ethyl ester, and gentisic acid indicate that these compounds are not teratogenic in rats even when given in amounts much greater than would be formed after ingestion of large doses of aspirin. The experiments reported here indicate, therefore, that salicylic acid is the causative agent in aspirin teratogenesis, and that its concentration and persistence can be influenced by a common environmental agent, benzoic acid.

DISCUSSION

As with most drug testing in animals, it is difficult to relate the effects of salicylates in laboratory animals to man. The reports of possible toxic effects of salicylates in human pregnancies (Jackson, '48; Earle, '61; Carter and Wilson, '63; Richards,

Fig. 4 Upper left, normal; other views, skeletons of animals after treatment at day 11 exhibiting fibular hemimelia and, in the most severe cases (lower right), absence of bones of the hindlimb. × 1.5.

'69) are at best only circumstantial evidence for salicylate embryotoxicity in man. However, the metabolic pathways of aspirin are the same in man and rats (Levy and Leonards, '66), and salicylates have been found to cross the human placenta (Jackson, '48). The fact that salicylates are not known to be teratogenic in rabbits is probably due to the different metabolism and rate of excretion (Bray et al., '48) and degree of salicylate binding to plasma protein in this species (Kucera et al., '68).

The therapeutic doses commonly used in man (4 g/day for analgesia, and 5–8 g/day for suppressing rheumatic inflammation, Woodbury, '68) are lower than the dosages used in the experiments reported here. Levy et al. ('69) reported that 7.2 g/day aspirin produced plasma salicylate levels in man of 39–42 mg %. Similar serum levels of 30–40 mg % measured in rats and reported here (fig. 6, at 6 and 12 hours) were produced by 500 mg/kg sodium salicylate, approximately five times the human dose. Therefore, the plasma or serum salicylate level is a much more accurate basis for comparison of the amount of drug available than the initial dose. The serum level mentioned above produced a strong teratogenic response in rats at day 9 and a moderate response at day 11. Thus, although information on the embryotoxicity of salicylates in man is inconclusive, the possibility of risk for women taking large therapeutic doses of aspirin during pregnancy should not be discounted in view of the present and previously reported animal studies.

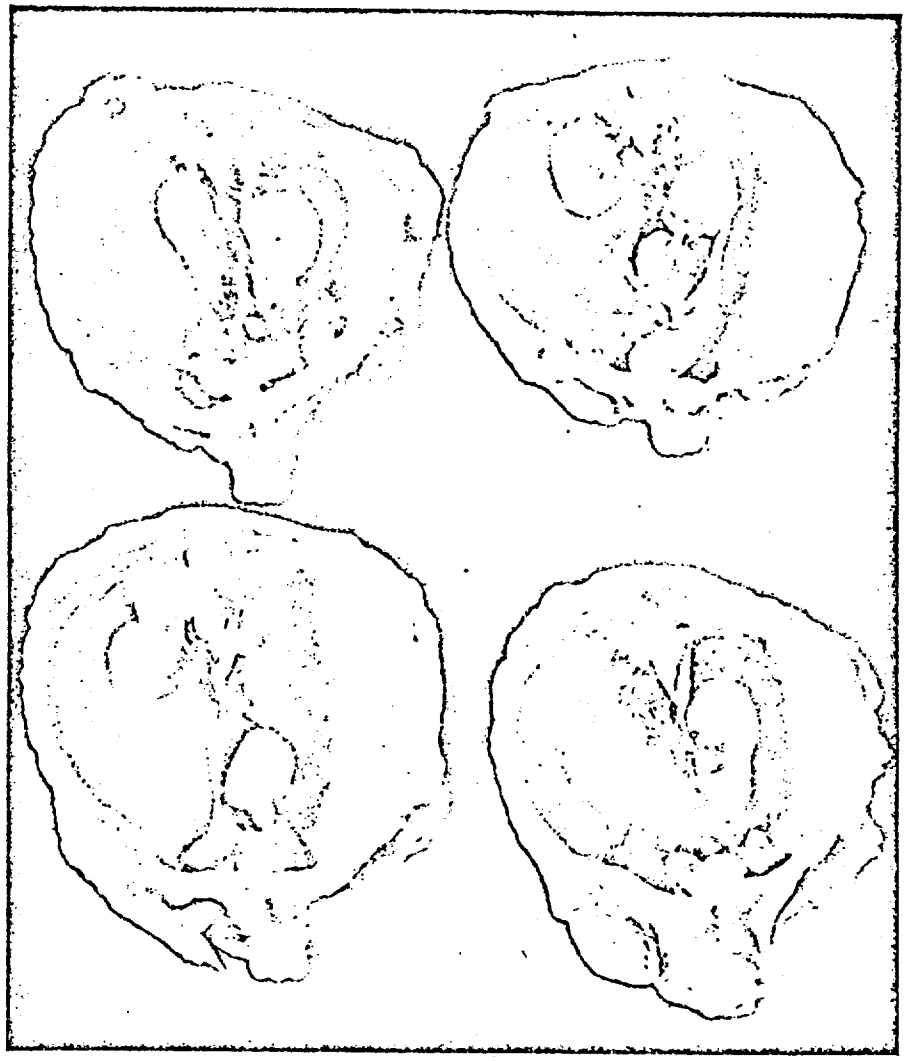

Fig. 5 Upper left, bilateral supernumerary kidneys; upper right, supernumerary kidney; lower left, ectopic kidney; lower right, fused supernumerary kidney. × 5.

TABLE 3

Augmentation of the teratogenicity of aspirin by pretreatment with benzoic acid at day 9 of gestation

Treatment	Dosage	No. of females	Total implants	% dead or resorbed	% abnormal survivors
	mg/kg				
Aspirin	250	5	60	3	21
Benzoic acid Aspirin	510 250	10	129	9	43
Aspirin	500	7	89	34	76
Benzoic acid Aspirin	510 500	10	124	34	91
Benzoic acid	510	7	88	0	3

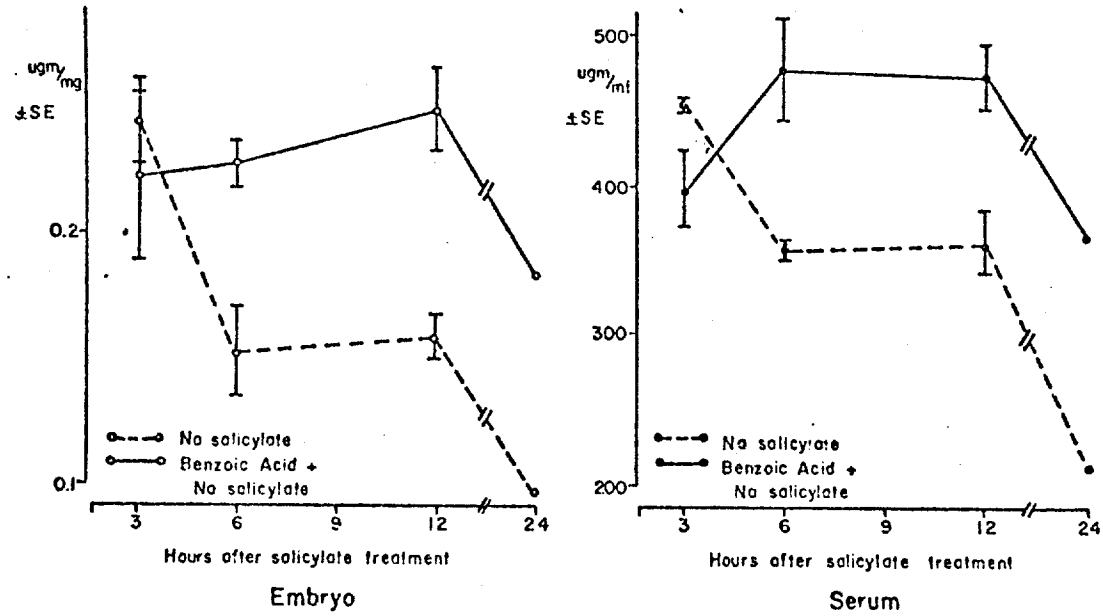

Fig. 6 Total salicylate concentration in embryos and serum with or without benzoic acid pretreatment.

The mechanism of teratogenic action of salicylates in the rat has not been elucidated in these experiments, but several facts have been established which should aid further studies on this problem. The site of teratogenic action of salicylates has been considered by several investigators, e.g., Goldman and Yakovac ('63), who felt that the action was indirect, the primary effect being on the maternal system. Experiments involving fluorometric determinations of salicylates presented here (fig. 6) showed that salicylates crossed the rat placenta and were thus available for direct action on the embryo. Results of the experiments with benzoic acid pretreatment indicated that salicylates might be acting directly on the embryo with maternal metabolic and excretory functions determining level and duration of embryonic exposure. However, neither maternally mediated nor direct embryonic action can be ruled out until an experiment is designed to eliminate one factor or the other completely.

By modifying the metabolism of aspirin and thus increasing the concentration and persistence of salicylic acid, benzoic acid effectively increased its biologic effect and pointed to salicylic acid as the causative agent. The interaction of these drugs has been demonstrated in man (Levy et al., '69); and although sodium benzoate is limited by law as a preservative in foods and beverages to one part per 1000 (Furia, '68, pp. 142–4) it is probably the preservative most widely used. By eating 1000 g or about 2 pounds of the right kinds of food a human being might consume as much as 1 g of sodium benzoate in a day. Levy et al. ('69) demonstrated almost total inhibition of salicyluric acid formation in man using 3.2 g of sodium benzoate. Since the body's capacity for making salicyluric acid is limited (Levy, '65) a smaller dose of benzoate might have a similar effect.

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197 (2157)

The synthesis and excretion of hippuric acid: The glycine factor.

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The rate of synthesis and excretion of hippuric acid in normal individuals and those with various renal disorder has already been studied by Kingsbury and Swanson[1] and this study made the basis of renal function test. In this work 2.4 grams of sodium benzoate was the dosage regardless of the size or weight of the individual. In the present paper the original work has

4 Churchman, J., *Jour. Exper. Med.*, 1913, xvii, 373; PROC. SOC. EXPER. BIOL. AND MED., 1922, xix, 288-31.

* A sample of pure H acid was kindly prepared for us by Drs. Derick and Strachan, of the laboratories of the National Aniline Company, and the bacterial cultures were furnished by Dr. J. F. Noble, to whom we extend our sincere thanks.

5 Bayer 205, *Science*, 1922, lvi, 514.

1 Kingsbury, F. B., and Swanson, W. W., *J. Biol. Chem.*, 1921, xlv, 4; *Arch. Int. Med.*, 1921, xxviii, 220.

been extended using dosages of sodium benzoate based on body weight in one series of 21 (50 milligrams per kilo) and on body surface in another series of 66 (1.8 grams per square meter of body surface). Body surfaces were obtained from the height-weight formula and chart of Dubois and Dubois.[2] The dosage based on surface was obtained by calculation after determining the body surface of one individual of average size and weight who had had 50 milligrams of benzoate per kilo of body weight. All individuals of both series were medical students who made the tests on themselves as part of their work in Physiolical Chemistry and whose urine specimens I made check analyses on by the method of Kingsbury and Swanson.[3] The benzoate was ingested quantitatively the first thing in the morning, having voided the night urine immediately before. Breakfast was omitted. The total water intake was 500 c.c. in every case. The urine was collected for the next three hours and immediately analyzed. The small and variable 3 hour normal output of hippuric acid independent of the ingested benzoate is included in these figures. Experience has shown that it is unnecessary to determine this and correct for it in the practical application of the test. In the first series of 21 it was noted that the groups of heavier individuals generally put out a smaller amount of hippuric acid per kilo of body weight than did the groups of lighter individuals, although all received the same dosage of sodium benzoate per kilo of body weight. This is shown in Table 1 in the form of percentages of excretion for the different weight groups.

This indicates that the actual amount of functioning tissue in the kidneys is less in its relation to body weight in the heavy person than in the light. This was also shown by a fairly high, but negative correlation coefficient 0.50, between the per cent. excretion and the body weight with a probably error of ±22 per cent.

In a second group of 66 normal individuals given benzoate, 1.8 grams per square meter of body surface, it was noted that the average percentage of excretion for groups of different body weights was nearly constant, indicating that the actual amount of functioning kidney substance is more nearly in proportion to the body surface than to the body weight. Calculations have shown that by using the square root of the body weight as a

2 Dubois and Dubois, *Arch. Int. Med.*, 1917, xvii.

3 Kingsbury, F. B., and Swanson, W. W., *J. Biol. Chem.*, 1921, xlviii, 13.

basis for the benzoate dosage that nearly the same amount is obtained for any one individual as by the body surface plan. This agrees with the results obtained by Austin, Stillman and Van Slyke,[4] who found that the square root of the body weight was more adequate than the body weight itself in their constant for urea excretion. The correlation coefficient between the milligrams of hippuric acid excreted per square meter of body surface and the volume of urine excreted on the same basis was small, 0.13, negative and with a very high probable error, ±64 per cent., indicating that in general there it little relationship between the excretion of hippuric acid and of water and that there is a slight tendency for the kidney that shows the best output of hippuric acid also to show the best concentrating ability. In the first group of 21 quite the reverse of this was true namely that the greater volume of water that was excreted in a test was accompanied by a greater relative amount of hippuric acid. This was well shown by correlating the milligrams of hippuric acid excreted per kilo of body weight with the volume of urine excreted on the same basis. The coefficient obtained was 0.42, positive, and with a probable error of ±28 per cent. A comparison of some of the findings with the two groups are shown in Table 1.

It will be noted from Table 1 that 91 per cent. of the individuals of the group of 66 were able to excrete more than 85 percent. of hippuric acid theoretically obtainable from the ingested benzoate. Values lower than 80 per cent. in this test for renal function should be regarded as indicating probable renal insufficiency.

Two normal individuals in a total of 89, given benzoate according to the body surface plan gave evidence that glycine was not furnished with sufficient rapidity for the synthesis and excretion of hippuric acid at the normal rate. The first, a healthy medical student, gave on two occasions tests of 53 and 67 per cent. He was thoroughly examined by Dr. George E. Fahr of the Department of Medicine, who also determined his Van Slyke[4] urea excretion constant. This was within the normal limits. The second student showed benzoate test figures of 69 on two occasions and while not examined physically was apparently in perfect health. Glycine in amount equivalent to the benzoate was

[4] Austin, J. H., Stillman, E., and Van Slyke, D. D., *J. Biol. Chem.*, 1921, xlvii, 91.

given with the benzoate to each of these students in subsequent tests. The first showed 90 per cent. and the second 84 per cent. It should be noted that there was an interval of at least a week between any two of these tests on the same individual. The benzoate dosage in each case was approximately 50 milligrams per kilo of body. Lewis and Griffith[5] gave rabbits the enormous dosage of 1 gram of sodium benzoate per kilo of body weight and found that the simultaneous administration of glycine markedly increased the rate of synthesis and elimination of hippuric acid. This was to have been expected since the dosage which they used was approaching the lethal dose of 1.7 grams per kilo. In previous tests using 2.4 grams of benzoate I had tried the effect of the simultaneous administration of glycine without result in the case of normal human beings, and I was therefore surprised that with the somewhat larger dose of 50 milligrams per kilo, but still a relative small dose, to find two persons out of a series of 89 who showed this peculiar condition. This apparent difficulty in certain persons of readily supplying glycine must be taken into account in making benzoate tests: Experiment with normal given benzoate per body surface basis shows administration of glycine increases rate of hippuric acid output in two hours' interval markedly but slightly in three hours interval. It will be necessary to supply sufficient glycine in all tests to rule out the possibility of an occasionally low benzoate test being due to this glycine factor. Gelatine can probably be adequately substituted for glycine for this purpose if given in proper amount and a short time before the administration of the benzoate to allow time for digestion.

TABLE I.

	Group 1, 21 cases		Group 2, 266 cases		Group 2		
Body wt. kilos.	No. of cases	Aver. H. A. % excreted	No. of cases	Aver. H. A. % excreted	% excretion	No. of cases	% of total
41- 50	1	100	1	96	80- 85	6	9
51- 60	5	91	16	91	86- 90	20	30
61- 70	11	90	26	96	91- 95	22	33
71- 80	3	83	21	91	96-100	18	28
81- 90	1	85	1	92			
91-100	0		1	100			

[5] Lewis, H. B., and Griffith, W. H., *J. Biol. Chem.*, 1923, lv, 22.

Acknowledgment of financial aid in conducting this work is made to the Graduate School of the University of Minnesota.

J. Lab. Clin. Med. 29(2): 188-196, 1944.

CLINICAL CHEMISTRY

REACTION FOLLOWING INGESTION OF SODIUM BENZOATE IN A PATIENT WITH SEVERE LIVER DAMAGE

Severe Substernal Pain, Shock, Increase in Icterus, Granulocytopenia and Eosinophilia in a Colored Male Following Hippuric Acid Excretion Test

Major Roy E. Kinsey, M. C., Army of the United States, and
Lt. Colonel Duward O. Wright, M. C., Army of the United States

THERE recently occurred in this hospital a reaction, following the ingestion of sodium benzoate for hippuric acid excretion test, in a patient with severe liver damage. This was of sufficient gravity to endanger his life. Three other patients with equally severe liver damage were given sodium benzoate simultaneously, from the same bottle, without untoward reaction. To test for impurities, six grams of the drug were given to a guinea pig but failed to cause any ill effect. In view of these findings and the generally accepted belief that sodium benzoate does not cause untoward systemic reactions, it was, at first, thought that the reaction was not due to the drug, so the test was repeated. This time there was an even more marked reaction, with severe substernal pain and shock, followed by granulocytopenia and an increase in the icteric index. The lack of case reports of severe reactions from sodium benzoate prompts the presentation of this case.

CASE REPORT

B. S. was a 24-year-old colored male admitted June 1, 1942, because of anorexia, postprandial epigastric distress, weakness, and dark urine, of four days' duration. His past health had been good and there had been no previous episodes of jaundice. He did not use alcohol or tobacco in any form. The admission examination revealed a well-developed and well-nourished colored male who appeared somewhat ill. There was moderate jaundice of the sclerae and mucous membranes. The heart and lungs were normal to percussion and auscultation, and the spleen and liver were normal to percussion and palpation. The stools were light, but contained some bile; the urine was quite dark and contained bile. The hemoglobin and red blood cell count were normal; white blood cell count, 5,850; differential: polymorphonuclear 60 per cent, eosinophiles 3 per cent, lymphocytes 32 per cent, monocytes 5 per cent; Kahn negative; icteric index, 57. The course of the patient's illness was gradually downhill for the first two weeks in spite of choline chloride, vitamin B complex, and diet rich in protein and carbohydrate, but after that he rapidly grew worse. On July 25, the patient was started on 6.4 mg. of vitamin K intramuscularly daily, because the prothrombin was 42 per cent of normal. By June 29, the liver dullness had diminished to 2 centimeters; the icteric index had mounted to 361; and a hemorrhagic tendency had de-

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Received for publication, Oct. 22, 1943.

veloped. The following morning, the patient was stuporous, and by afternoon was comatose. Marked bleeding now occurred after cleaning his teeth or after any minimal trauma to the mouth.

By the use of transfusions from donors who had received vitamin K, whole blood transfusions, plasma, vitamin K, and other measures, the bleeding was controlled and the patient improved. The prothrombin increased to near normal, the icteric index gradually diminished to 220 by July 15, and the patient appeared improved. On July 21, it was decided to attempt a hippuric acid excretion test in an effort to determine the functional capacity of the liver. The routine test, as devised by Quick,[2] was used, the patient being given 6 Gm. of sodium benzoate in 30 c.c. of water, by mouth. The test was uneventful until four hours after the administration of the sodium benzoate, when the patient suddenly complained of severe pain across his upper chest, "as if somebody was stabbing me with a knife," extreme dyspnea, and orthopnea. His blood pressure earlier in the morning was 106/70; but now it was 150/120 and the pulse was rapid and thready. The patient rapidly went into shock, with the pulse becoming imperceptible, and lapsed into a semicoma; then by the use of plasma, oxygen, and stimulants, he recovered from the shock and felt better within a few hours. Electrocardiogram, x-ray of the chest, physical examination, urine, chemical studies of the blood, and other examinations were all essentially as before the test, except that the icteric index had gone up to 275. The following day, on July 22, the patient's condition was further improved, and repeated studies failed to reveal a cause of the episode.

A rather rapid survey of the literature revealed no report of untoward systemic reactions from sodium benzoate, and it was thought that the episode was incidental and not due to the drug. The first test was not completed, and as the condition of the patient was satisfactory, the test was repeated on July 23, the patient again being given 6 Gm. of sodium benzoate in 30 c.c. of water, by mouth. At approximately the same time, four hours after ingesting the chemical, the patient again complained of severe chest pain, this time across the lower chest. Extreme dyspnea and orthopnea occurred a few minutes later, with the blood pressure rising to 155/126. The patient again rapidly went into shock, requiring intravenous glucose, plasma, oxygen, and stimulants for relief. During the shock his condition resembled that of a patient with a rather large pulmonary embolus. He could not be aroused for about twenty minutes and was stuporous and confused for four hours. Again the patient made a recovery from the shock, but this time only partially regained his former condition, feeling much weaker, appearing rather ill, and lacking his usual appetite and good spirits. Again, a thorough study was essentially as before the reaction, except for the icteric index, which had increased from 220 on July 15 to 275 on July 22, and to 285 on July 24. This was interpreted as an indication of increased damage to the liver, as there was no evidence of hemolysis of a large number of red blood cells. After the second episode, it was felt that both reactions were due to the sodium benzoate. This was further substantiated by the fact that the patient had received only vitamin K, vitamin B complex, choline chloride, transfusions, and plasma during his hospital stay, and continued to receive these during his recovery from each episode. The two reactions were essentially similar and occurred at almost exactly the same time after ingesting the drug.

After the first reaction, the white count was 4,450, with 40 per cent polymorphonuclears, 8 per cent eosinophiles, 7 per cent basophiles, and 46 per cent lymphocytes.

On July 26 he developed a cough, fever, sore throat, and malaise. Examination revealed mild redness of the throat and enlarged cervical glands. Otherwise, nothing was made out on physical examination aside from jaundice, small liver, and emaciation. The white blood cell count was 1,600, with 4 per cent eosinophiles, 1 per cent basophiles, 61 per cent lymphocytes, and 34 per cent monocytes. Repeated examinations of several smears showed no neutrophilic granulocytes. Tables I and II tabulate the white and differential blood counts, hemoglobin, red blood cell counts, liver, and transfusions during this period. One hundred units of liver extract and a 250 c.c. transfusion of whole citrated blood were given. On the following day, July 27, there were 2,400 white cells, again without any neutrophiles, the differential showing 5 per cent eosinophiles, 2 per cent basophiles, 58 per cent lymphocytes, and 35 per cent monocytes. Several checkup counts, including the various vital

TABLE I

BLOOD STUDIES

	DATE	HGB.	R.B.C.	W.B.C.	DIFFERENTIAL								
					MYELOB.	MYELOS.	JUV.	STABS.	SEGS.	EOS.	BAS.	LYMPHS.	MONOS.
1	6/ 2/42	92%		5,580	0	0	0	0	60	3	0	32	5
2	7/23/42	85%		4,450	0	0	0	0	40	8	7	45	0
3	7/26/42*			1,600	0	0	0	0	0	4	1	61	34
4	7/27/42*			2,400	0	0	0	0	0	5	2	58	35
5	7/28/42 A.M.	80%	4.08	2,650	0	0	0	0	0	4	1	60	35
6	(A.T.) P.M.	100%	4.58	3,200	0	0	0	0	2	9	0	39	50
7	7/29/42 A.M.	104%	5.50	4,700	0	0	2	2	1	5	2	74	15
8	P.M.			5,000	0	0	0	0	0	6	0	51	43
9	7/30/42 P.M.*	95%	4.14	5,000	6	30	0	0	9	7	2	31	14
10	7/31/42 A.M.	95%	4.72	7,050	1	30	4	4	13	8	2	24	15
11	P.M.	100%	4.71	7,350	1	12	1	14	11	7	4	41	9
12	8/ 1/42 A.M.	100%	5.33	7,500	0	10	3	6	30	17	2	28	4
13	(A.T.) P.M.		5.32	8,300	0	2	0	12	30	9	1	33	13
14	8/ 2/42	95%	5.20	8,150	0	2	5	11	23	6	0	40	13
15	8/ 3/42 A.M.	95%	5.50	6,500	0	0	3	8	38	6	1	31	13
16	P.M.			6,150	0	2	4	7	35	5	1	34	12
17	8/ 4/42 A.M.			8,350	0	0	0	0	62	1	2	35	0
18	P.M.			11,100	0	2	3	8	37	6	2	29	13
19	8/ 5/42	85%		14,700	0	4	6	11	49	2	1	24	3
20	8/ 6/42	90%	5.15	10,200	0	3	1	2	54	5	2	28	5
21	8/10/42 A.M.			20,350	0	0	0	0	91	0	1	9	0
22	P.M.	95%		36,600	0	0	2	27	66	0	0	5	0
23	8/11/42			18,950	0	0	0	25	70	0	0	5	0
24	8/12/42			14,100									
25	8/13/42			10,050	0	0	0	10	62	11	2	9	6
26	8/14/42			9,950	0	0	0	7	61	5	2	18	7
27	8/15/42			13,350	0	1	1	1	62	8	2	22	3

*Mean average of 9 complete counts.

A.T.—After Transfusion.

stains, continued to show complete absence of the neutrophilic series. The patient continued to receive whole blood transfusions, 200 units of intramuscular liver daily, and large amounts of all the vitamins.

On July 28, the white count was 2650 and was still without any of the neutrophilic series. The patient was much worse, the cough and fever had increased in severity, and the throat was now quite red and swollen, but without ulcers. The cough was productive of a greenish-yellow, foul sputum, with innumerable Vincent's organisms, but with no pus cells.

By July 29, the patient had not improved, and a sternal biopsy by the punch method was made. This revealed an arrested maturation of the myeloid elements of the bone marrow, except eosinophiles and basophiles. Peculiarly enough, these two constituents were found in normal or increased numbers, mostly in mature form. The amount of intramuscular liver injected was increased, and the small blood transfusions were given more frequently. The blood count gradually increased after this so that forty hours after the increase in liver dosage, the total white count was 5000, with 6 per cent myeloblasts, 30 per cent myelocytes, 9 per cent segmented neutrophilic forms, 7 per cent eosinophiles, 2 per cent basophiles, 32 per cent lymphocytes, and 14 per cent monocytes.

The bone marrow apparently regained its normal ability to maturate granulocytic neutrophiles, since, when the patient developed a severe acute systemic infection on Aug. 10, the white blood count rapidly rose to 36,600, with 95 per cent granulocytes. The infection was controlled by the use of sulfadiazine, without effect on the bone marrow, but he eventually died on Sept. 11, 1942, from liver failure. The icteric index gradually decreased from 285 to 200, and remained at approximately this level until death. Autopsy revealed severe yellow atrophy of the liver, marked degeneration of the corpus striatum of the brain, marked wasting of the striated muscles, and a normal bone marrow.

DISCUSSION

There are many reports of the use of sodium benzoate in the treatment of the rheumatic state, and more recently for the determination of hepatic function in the hippuric acid excretion test. Bryan[1] in 1925 stated "A review of the literature indicated that normal human beings may completely convert at least 25 grams of sodium benzoate to hippuric acid without toxic effect and with the appearance of only traces of free benzoic acid in the urine." Quick[2] felt that sodium benzoate was a safe drug in moderate amounts when he devised the hippuric acid excretion test in 1933. Snell and Magath[3] feel that the hippuric acid excretion test is a test of the detoxifying function of the liver and is based on the conjugation by the liver of amino-acetic acid and benzoic acid into hippuric acid, but mention no harmful effect from its use. Bartels[4] repeated the test three times on most of 148 cases with thyroid disease, without untoward effect. Hirschheimer[5] did 246 tests on pregnant women, some complicated and others uncomplicated, without ill effect. The *Journal of the American Medical Association*[6] stated, "There is no direct or indirect evidence to indicate that sodium benzoate has any toxic effect on the liver. The drug was formerly used for the treatment of rheumatic disease and has been given to human beings in doses as large as 25 to 60 grams a day without producing any harmful effect. Quick's hippuric acid test has been performed on many patients with extreme degrees of injury to the liver without any appreciable toxic effects or without depressing other hepatic functions; slight gastric irritation has been noted in a few cases, but these symptoms are of a temporary nature." Quastel and Wales[7] found that there was low excretion of hippuric acid after both oral and intravenous administration of sodium benzoate in schizophrenia, but mentioned no ill effects. Davies, Wales, and Hughes[8] were of the same opinion and mentioned seventy-five cases

without complications. Londe and Probstein[9] performed a modified hippuric acid test in many children without ill effect. Finkelman, Hora, Sherman, and Horwitt,[10] Campbell,[11] Vaccarro,[12] Snell and Plunkett,[13] Lipshutz,[14] Quick,[15] Haines, Magath, and Power,[16] and many others reported using this test in numerous cases, and none mentioned systemic reactions.

The absence of neutrophiles in the peripheral blood in this case, known by various names such as granulopenia, granulocytopenia, agranulocytosis, agranulocytic angina, leucopenia, or neutropenia, and which was described originally by Schultz[17] in Germany in 1922, and then by Lovett[18] in America in 1924, has not been described following ingestion of sodium benzoate. During the past twenty-one years, there have been numerous case reports and discussions, which have shown that with the increase in use of amidopyrine, arsphenamine, sulfonamides, and other substances depressing to the bone marrow, the incidence of granulocytopenia has increased. While a great many of these cases occur spontaneously, it is interesting to note that in most case histories there has been a preceding treatment of some type, by either a dentist or a physician. In Kracke's[19] series of 473 patients, nearly ¾ had been receiving treatment for some other disease for a variable period prior to the attack of granulocytopenia.

Granulocytopenia in the various races has been discussed, and it has been found to be rare in the colored race, occurring eight times in the 473 cases collected by Kracke.[19] A review of the literature further reveals that it occurs in a ratio of about two female patients to one male.

The cause of granulocytopenia has been discussed at great length. The first investigators directed their efforts on the hypothesis that the cause was some type of infection, because various organisms were usually cultured from the lesions in the throat and blood stream. Also, Fried and Dameshek[20] were able to produce a type of primary granulocytopenia in rabbits by the injection of *Salmonella suipestifer*. Kracke[19] doubts that Fried and Dameshek reproduced a true granulopenic picture, but produced the picture which is commonly seen following injections of any killed organism. The more recent work is directed toward the fact that granulocytopenia is a disease of the bone marrow, and that the basic picture is the disappearance of the neutrophiles from the peripheral blood, which, in turn, is followed by the invasion of any and every organ that is accessible by organisms. According to Jackson (Cecil's Text),[21] the eosinophiles disappear early and reappear late. The total number of lymphocytes usually is reduced. Pus does not form during this stage. Reznikoff[22] feels that the following four factors are important in causing this disease: fatigue, drugs, menstruation, and infection. In the thirteen cases studied by him, he found that fatigue due to excessive work, lack of sleep, and worry was more frequently encountered than any of the other etiologic conditions, and it was also his opinion that monocytosis is an evidence of good prognosis when it occurs early and is persistent.

While the majority of cases are due to toxic action of a drug on the bone marrow, a smaller group is due to the toxic effect of an infection on the bone marrow, as for instance, typhoid fever, influenza, and measles, which are associated with a leucopenia. There are probably three causes: (1) a primary form with fever, ulcerative mouth lesions, and agranulocytosis, (2) malignant granulocytopenia of bacterial origin, and (3) malignant granulocytopenia of toxic origin. Ralph U. Leser,[23] in discussing agranulocytic angina due to the sulfon-

amide drugs, suggests treatment by parenteral liver therapy and by mouth, if possible; repeated daily transfusions of 200 to 300 c.c.; pentnucleotides; a high caloric diet; good nursing; adequate cardiac and respiratory stimulation; withdrawal of all analgesic drugs containing the benzene ring, and also all the so-called barbiturates. He feels that adenine sulfate is quite valuable and that local treatment of the oral lesions is important. Ottenberg[24] used transfusions from donors who had received typhoid vaccine to stimulate leucocytosis in treating leucopenia. He reports only one case and believes that the cure was unquestionably due to the treatment. He feels that this is the result not of the actual number of leucocytes transfused, but to the passive transmission of the bone marrow-stimulating factor. Dameshek and Wolfson[25] treated two cases of severe agranulocytosis with sulfathiazole in addition to transfusions, pentose nucleotides, and liver extract, feeling that the recovery, at least in part, was due to the effect of the sulfonamide drug on the sepsis, which was almost certainly present.

TABLE II

TOTAL NUMBER OF WHITE CELLS OF EACH CLASS, AND TREATMENT

	W.B.C.	NEUTROPHILES	EOSINOPHILES	BASOPHILES	LYMPHS.	MONOS.	TREATMENT	
							TRANSFUSIONS	LIVER IN UNITS
1	5,850	3,510	176	0	1,872	292		
2	4,450	1,780	356	312	2,002	0		
3	1,600	0	64	16	976	544	250 c.c.	100 units intramus.
4	2,400	0	120	48	1,392	840	250 c.c. A.M. & 250 c.c. P.M.	200 units intramus.
5	2,650	0	106	26	1,590	928	250 c.c.	100 units intramus.
6	3,200	64	288	0	1,248	1,600	250 c.c.	100 units intramus.
7	4,700	188	235	94	3,478	705	250 c.c.	200 units intramus.
8	5,000	0	300	0	2,550	2,150	250 c.c.	200 units intramus.
9	5,000	2,250	350	100	1,600	700	250 c.c. A.M. & 250 c.c. P.M.	400 units intramus.
10	7,050	3,666	564	141	1,622	1,057	250 c.c.	200 units intramus.
11	7,350	2,866	515	294	3,014	661	250 c.c.	200 units intramus.
12	7,500	3,675	1,275	150	2,100	300	250 c.c.	200 units intramus.
13	8,300	3,652	747	83	2,739	1,079	250 c.c.	200 units intramus.
14	8,150	3,341	489	0	3,260	1,060	500 c.c.	400 units intramus.
15	6,500	3,185	390	65	2,015	845	250 c.c.	200 units intramus.
16	6,150	2,952	307	62	2,091	738	250 c.c.	100 units intramus.
17	8,350	5,146	83	166	2,905	0		100 units intramus.
18	11,100	5,550	666	222	3,219	1,443		100 units intramus.
19	14,700	10,290	295	147	3,528	441		100 units intramus.
20	10,200	6,120	510	204	2,856	510	500 c.c.	100 units intramus.
21	20,350	18,315	0	203	1,832	0	500 c.c.	
22	36,600	34,770	0	0	1,830	0	500 c.c.	
23	18,950	18,005	0	0	945	0	500 c.c.	
24	14,100						500 c.c.	
25	10,050	7,200	1,100	200	900	600		
26	9,950	6,732	495	198	1,832	693		
27	13,350	8,512	1,064	266	3,109	399		

Apparently the bone marrow varies in its appearance during the various stages of the disease, and there are at least two types of bone marrow seen in the early stages, one in which there is myeloid hypoplasia, and another in which there is arrest in maturation of the myeloid elements. From the literature one would assume that where myeloid hypoplasia exists, the prognosis is poor, whereas in those in which there is arrest of maturation of the myeloid elements, the outlook is better. Boyd[26] states "The bone-marrow varies in the fatal cases. As a rule it is aplastic and appears to be completely exhausted, as

is seen in experimental benzol poisoning. In others it is normal, and in still others it may be hyperplastic. In these latter cases there would appear to be a blockade of the marrow or a lack of maturation of the myelocytes, thus preventing the mature leucocytes from entering the circulation, a state of affairs comparable with what is found in pernicious anemia."

Kracke and Parker[27] in 1938 thoroughly reviewed the literature of drug therapy in relation to the neutropenic states, and cited eleven cases of granulocytopenia, nine with fatal termination resulting from the use of sulfanilamide. In all instances a large amount of the drug was administered over a relatively long period before the condition made its appearance. Rinkoff and Spring[28] in 1941 reported six cases illustrating the toxic effect of sulfonamides on the myeloid elements, three due to sulfanilamide, two to sulfapyridine, and one, a combination of sulfapyridine and neoprontosil. Three of their patients developed granulocytopenia within the first twenty-four hours or less of therapy, and three, after fourteen days or more of therapy. Three of these cases developing within twenty-four hours would mitigate against Kracke's experience that prolonged therapy with sulfanilamide was necessary to induce granulocytopenia.

The reactions occurring in our patient were probably of the so-called "anaphylactoid type." This term is used here to indicate a reaction closely resembling anaphylactic shock, but occurring after a chemical. The predominating symptoms of severe chest pain, extreme dyspnea, and orthopnea, early increase in blood pressure with later fall, rapid thready pulse, and shock, indicate the more common cardiac rather than pulmonary type of reaction. There apparently was no desensitization after the first reaction, as the second was as severe as the first. The increase in icteric index could be interpreted as the result of the anaphylactoid reaction, or as the direct result of chemical poisoning.

SUMMARY

This case presented various interesting problems. The patient apparently had a reaction following his first dose of sodium benzoate with some increase in his icteric index, probably due to further damage of the liver, and a second dose forty-eight hours later again caused a severe reaction and probably further increased the liver damage. The combination of the two doses produced an arrest of the maturation of the neutrophilic granulocytes in the bone marrow, but allowed the basophiles and eosinophiles to maturate. Another patient with acute hepatitis and an even higher icteric index had no reaction from either the first or second test, performed at the same time as the case being reported here.

Liver damage, or increase in damage to an already diseased liver, following sodium benzoate, must be extremely rare, as we have been unable to find any report of cases in the literature. The increase in jaundice and the increase in general debility of the patient could be interpreted as indicating further damage to the liver either as a direct result of the chemical or as a result of the reaction.

As to the bone marrow and peripheral blood, the situation was also peculiar in that it is customary for the eosinophiles and basophiles to disappear from the peripheral blood, and their maturation to be arrested along with the

other granulocytes, but in this case they appeared in normal to increased numbers in the peripheral blood, and apparently matured normally in the bone marrow. It is impossible to say what part, if any, the severely damaged liver played in the granulocytopenia, except that there was response to very large doses of liver. A point against the liver having any particular part is the fact that later there was a white count of 36,600, secondary to a severe septicemia, showing that the bone marrow could respond even though a badly damaged liver condition was still present. Further proof that the liver probably played little part was the fact that the bone marrow was normal at autopsy even though very few normal liver cells remained.

CONCLUSION

1. A colored male with marked yellow atrophy of the liver had a severe reaction on two occasions, approximately four hours after ingesting sodium benzoate, manifested by excruciating substernal pain, elevation of blood pressure, dyspnea, and orthopnea, followed by shock.

2. Three days after ingesting the second dose of sodium benzoate, the patient developed signs of systemic infection and the white blood count showed an absence of the neutrophilic granulocytes, but a normal to increased number of eosinophiles and basophiles.

3. The bone marrow showed arrest of maturation of the neutrophilic myeloid elements, but normal maturation of the eosinophiles and basophiles.

4. There was later a marked leucocytic response to a generalized septicemia, showing complete recovery of the bone marrow.

5. The icteric index increased from 220 to 275 after the first reaction, and to 285 after the second reaction. The cause of the increase in icteric index after the reactions remains undetermined.

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Competitive inhibition of D-amino acid oxidase by benzoate as a function of substrate

For present purposes competitive inhibition may be summarized briefly as follows: The enzyme, E, bears a site at which either substrate, S, or inhibitor, I, combines reversibly to form an enzyme-substrate complex, ES, or an enzyme-inhibitor complex, EI, respectively. The reactions involved are $E + S \rightleftharpoons ES \rightleftharpoons E + P$ (a), where P is the product derived from S by enzyme action, and $E + I \rightleftharpoons EI$ (b). The quantitative relationships between the components involved in (a) are expressed by $(E)(S)/(ES) = K_s$ (c), and $(E)(I)/(EI) = K_i$ (d), where K_s and K_i are constants and the parentheses indicate concentrations. The constants are usually evaluated by means of the equation $1/v = [1/V_m][K_s + K_s(I)/K_i][1/(S)] + 1/V_m$ (e), where v, which is considered directly proportional to (ES), is the rate of formation of P in the presence of rate-limiting (S) and V_m is the maximum rate. Plots of $1/v$ against $1/(S)$ gives straight lines with the same intercept of the $1/v$ axis. The intercept is $1/V_m$. When I is not present the second term within brackets equals zero and the slope of the line measures K_s. In the presence of I the slope is a function of both constants. Conformation of experimental data to (e) is conventionally taken to indicate competitive inhibition.

The foregoing implies that the value of K_i should be independent of the particular substrate used in its estimation. However, in the present work, which has been reported in preliminary form[1], the competitive inhibition of D-amino acid oxidase by benzoate[2,3] was studied as a function of different substrates and, contrary to expectation, the value of K_i was found to depend upon the substrate used in the estimation of the constant.

Aqueous extracts of acetone-dried pig kidney were used as preparations of the oxidase. The D- and DL- form of each amino acid tested was used, except for the proline which was employed as the DL-mixture only. Two samples of each substrate were used; one was obtained from Nutritional Biochemicals Corporation, the other from California Corporation for Biochemical Research. The oxidation of substrate by the enzyme was followed manometrically[4].

In a number of tests four levels of substrate alone and three levels of inhibitor in the presence of three of substrate were employed. Estimations of K_s and K_i were made conventionally from plots of $1/v$ against $1/(S)$. In other tests three levels of substrate alone and three levels of inhibitor plus one of substrate were employed. In these tests two amino acids were studied simultaneously. In addition to the constants, these tests yielded relative values of V_m. Representative tests of the first kind are given in Fig. 1. These tests, by usual interpretation and in agreement with earlier tests with alanine[2,3], indicate that benzoate inhibits the oxidase by competing with substrate.

The means of all values of K_s, K_i, and relative values of V_m are given in Table I. At least three levels of K_i are apparent, one obtained with alanine or proline, another with valine, isoleucine, or phenylalanine, and a third with methionine. The levels of K_i do not appear correlated with the corresponding values of K_s or V_m.

The present study does not provide definitive explanation of the variation of K_i with substrate. However, one possibility bears consideration: The inhibitor may react only with the enzyme-substrate complex with the liberation of substrate, *i.e.*, instead of (b), reaction $ES + I \rightleftharpoons EI + S$ (f) occurs. This reaction in contrast to (b) implies that the formation of EI depends upon the particular ES involved. If (f) obtains, the inhibitor constant is described by $(ES)(I)/(EI)(S) = K_{is}$ (g), rather than (d). The equation relating rate of reaction to (S) is then the same as (e) except that the second term within the brackets, $K_s(I)/K_i$, is replaced by $(I)/K_{is}$. If both (b) and (f) occur, two species of enzyme inhibitor complex are implied, and the second term is $(I)[K_s/K_i + 1/K_{is}]$. Since each of the terms reduce to $(I)/c$, where c is a constant, the same rate equation applies to each possibility. It follows from this that data conforming to conventional requirements for competitive inhibition, but obtained by use of one substrate, can not define the reaction(s) involved in the inhibition. If more than one substrate is used and the values of K_i obtained are all the same, it is reasonable to infer that reaction (b) is involved. If K_i varies with substrate, it seems reasonable to infer that reaction (f) occurs. The present results with the

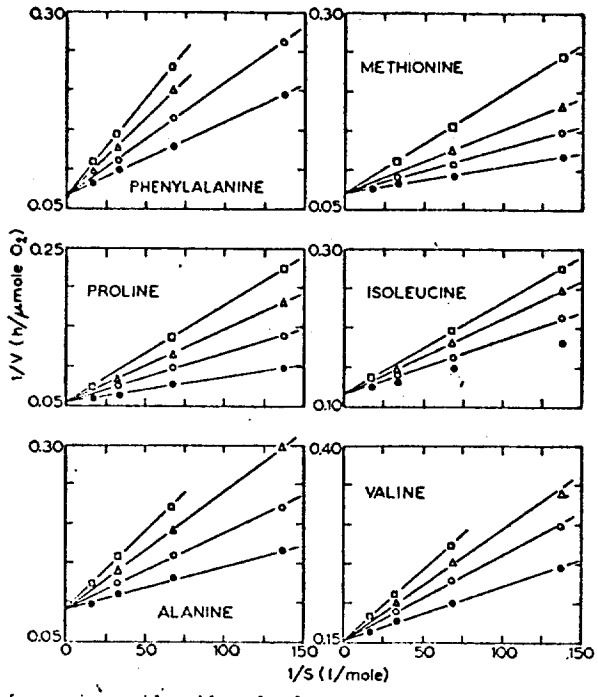

Fig. 1. Inhibition of D-amino acid oxidase by benzoate. The solid circles indicate the results obtained in the absence of inhibitor. The open circles, trangles, and squares indicate respectively the presence of 10^{-4}, $2\cdot10^{-4}$, and $4\cdot10^{-4}$ M benzoate in the case of methionine and $5\cdot10^{-5}$, 10^{-4}, and $1.5\cdot10^{-4}$ M benzoate in the tests with the other amino acids.

TABLE I

COMPETITIVE INHIBITION OF D-AMINO ACID OXIDASE BY BENZOATE AS A FUNCTION OF SUBSTRATE

The values given are means. The values of K_s and K_i bear standard deviations of about 5%.

Amino acid	*Relative* V_m	K_s (*M*)	K_i (*M*)
Alanine	1.00	$6.3\cdot10^{-3}$	$5.9\cdot10^{-5}$
Proline	1.66	$5.8\cdot10^{-3}$	$7.0\cdot10^{-5}$
Phenylalanine	1.39	$14.0\cdot10^{-3}$	$9.2\cdot10^{-5}$
Valine	0.60	$4.6\cdot10^{-3}$	$9.6\cdot10^{-5}$
Isoleucine	0.79	$4.1\cdot10^{-3}$	$10.4\cdot10^{-5}$
Methionine	1.32	$5.3\cdot10^{-3}$	$16.3\cdot10^{-5}$

oxidase appears to demand reaction (f). However, there is indication that the oxidase combines with competitive inhibitors in the absence of substrate[5]. Therefore, both reaction (b) and (f) may occur.

Some support for the above considerations is provided by the non-competitive reversible inhibition of hexokinase by glucose-6-phosphate[6]. As indicated for benzoate, the value of the inhibitor constant for the phosphate was found to depend upon the substrate used in its determination. Discussion of mechanisms that may account for the finding is presently not in order; however, the finding does suggest reaction between the phosphate and kinase substrate complex and that the extent of the reaction depends upon the substrate involved.

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This work was supported by the U.S. Atomic Energy Commission.

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Received May 1st, 1959

J. Biol Chem. 138(2): 507-12, 1941

INHIBITION OF THE *d*-AMINO ACID OXIDASE BY BENZOIC ACID

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(Received for publication, November 18, 1940)

Benzoic acid has been reported to inhibit the oxidation of succinic acid by muscle (1), the oxidation of butyric acid and crotonic acid by liver slices (2), and the oxidation of acetoacetic acid by kidney slices (3). It has also been reported to inhibit the oxygen uptake of liver and kidney slices, diaphragm, minced liver, kidney, and brain (4).

In the present work it was found that benzoic acid had a slight inhibitory effect on the oxygen uptake of broken cell preparations of rat liver and kidney. It had no effect on the oxidation of succinic acid, lactic acid, hypoxanthine, xanthine, uric acid, choline, tyramine, *l*(−)-tyrosine, *l*(−)-proline, *l*(−)-phenylalanine, sarcosine, or ethyl alcohol by the liver preparation. The oxidation of *d*-amino acids by various preparations of the *d*-amino acid oxidase was markedly inhibited by benzoic acid.

EXPERIMENTAL

Effect of Benzoic Acid on Oxidation of d-Amino Acids—The oxidation of *d*-amino acids by rat liver and kidney slices and by the preparations of the *d*-amino acid oxidase described below and the effect of benzoic acid on the oxidation were tested by measuring oxygen uptakes manometrically. The measurements were made with the usual Warburg apparatus at 37.5°.

The extra oxygen uptake of liver slices in the presence of 0.0075 M *d*(−)-alanine was 30 microliters per 30 minutes per 100 mg. of wet tissue. This extra oxygen uptake was completely inhibited by 0.001 M benzoic acid. The presence of glycine did not affect the inhibitory effect of benzoic acid. The benzoic acid did not

inhibit the oxygen uptake of the liver slices in the absence of the amino acid. The extra oxygen uptake of kidney slices in the presence of 0.0075 M *d*(−)-alanine was 53 microliters per 30 minutes per 100 mg. of wet tissue. 0.001 M benzoic acid inhibited the rate 50 per cent. The oxygen uptake of the kidney slices in the absence of the substrate was inhibited 6 per cent by benzoic acid.

The oxidation of 1 mg. of *d*(−)-alanine, *d*(+)-phenylalanine, and the *d* isomer of *dl*-proline by broken cell preparations (5) of rat liver and kidney and the washed proteins (6) of rat liver was completely inhibited by 0.01 M benzoic acid at pH 7.0, 7.5, and 8.0. With 0.001 M benzoic acid the rate of oxidation of these amino acids was inhibited about 90 per cent. In most cases the oxygen uptake of the broken cell preparations alone was inhibited about 10 per cent at pH 6.6 and 5 per cent at pH 8.1. The inhibition usually decreased as the pH approached 7.4, and then increased up to pH 8.1. In a few experiments the inhibition was negligible at pH 6.6 to 8.1.

Benzoic acid inhibited the activity of two purified preparations of the *d*-amino acid oxidase. One preparation was made by extracting 1 gm. of dry pig kidney (7) with 10 ml. of water, and then centrifuging. 1 ml. of the supernatant liquid plus 1 ml. of 0.1 M pyrophosphate buffer, pH 8.3, was used to oxidize the amino acids. The other preparation was a reconstructed oxidase composed of 3 to 6 mg. of a preparation of the protein fraction of the oxidase plus 2.4 to 4.8 γ of flavin-adenine dinucleotide. The protein preparation was made from the dry pig kidney and the flavin from yeast (7). Neither preparation of the oxidase could oxidize *l*-amino acids.

Data illustrating the effect of benzoic acid on the oxidation of *d*-amino acids by the reconstructed oxidase are given in Fig. 1. The data show that 10^{-4} M benzoic acid inhibits the activity 79, 52, and 50 per cent in the case of alanine, methionine, and proline respectively. The extent of inhibition is calculated from the oxygen uptakes at 10 minutes. In addition, the rate of oxidation of 1 mg. of *d*(+)-phenylalanine, *d*(+)-leucine, and *d*(+)-tyrosine was found to be inhibited 57, 28, and 64 per cent by 10^{-4} M benzoic acid. The data for methionine in Fig. 1 show that the extent of oxidation was not affected by benzoic acid. This was

also true in the case of the other amino acids when the experiments were of longer duration. Similar data were obtained with the kidney extract. The benzoic acid also inhibits the rate of reduction of methylene blue by mixtures of the oxidase preparations and amino acids.

It can be shown from the data in Fig. 1 that the oxidation of the amino acids in the presence and absence of benzoic acid is a first order reaction; *i.e.*, a straight line is obtained when the logarithm of the concentration of unoxidized amino acid is plotted as a function of time. This indicates that the enzyme is not pro-

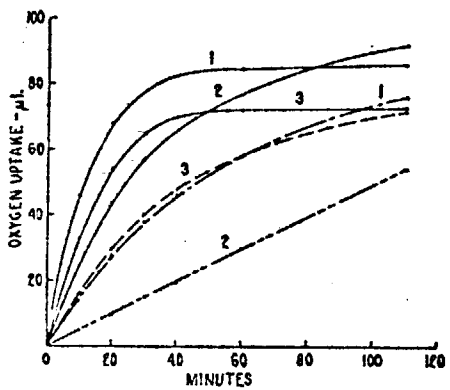

FIG. 1. Effect of benzoic acid on the activity of a reconstructed *d*-amino acid oxidase. The continuous lines represent oxidation of amino acids in the absence of benzoic acid; broken lines, oxidation in the presence of 10^{-4} M benzoic acid. The oxidase consisted of 3 ml. of the protein preparation and 2.4 γ of flavin-adenine dinucleotide in 2 ml. of 0.05 M pyrophosphate buffer, pH 8.3. Curves 1, 0.0038 M *dl*-proline; Curves 2, 0.0056 M *dl*-alanine; Curves 3, 0.0034 M *dl*-methionine.

gressively inhibited by benzoic acid. This is also indicated by the fact that incubation of the enzyme and benzoic acid for 30 minutes before the addition of the amino acid does not affect the extent of inhibition.

A comparison of the effect of benzoic acid and that of other active acids is given in Table I. Under the same conditions isophthalic, hippuric, nicotinic, quinoline-β-carboxylic, and pyrazinecarboxylic acids were slightly active (0.08 or less), while *p*-aminobenzoic, phthalic, phenylacetic, picolinic, quinolinic, dinicotinic, 6-hydroxynicotinic, pyrimidine-4-carboxylic, acetic,

iodoacetic, stearic, and malonic acids, and benzamide, aniline, phenol, toluene, benzene, nicotinamide, trigonelline, pyridine, and glycine were inactive. These data show that compounds containing the benzene ring are more effective than those containing other rings, that the carboxyl group is necessary for inhibition, and that further substitution in the ring, or between the ring and the carboxyl group, depresses the effectiveness of the compound.

The following observations indicate that the inhibition produced by benzoic acid is reversible. (*a*) The inhibitory effect disappeared after dialysis. (*b*) Preparations of the protein

TABLE I

Comparison of Inhibitory Effect of Benzoic Acid on d-Amino Acid Oxidase with Effect of Other Compounds

The activity of the kidney extract was tested in the presence of 2 mg. of *dl*-alanine. The decrease in activity produced by each substance was calculated from the oxygen uptakes at 10 minutes. The measure of inhibition given in the table is the ratio of the decrease in activity produced by the substance in 10^{-4} M concentration to that produced by 10^{-4} M benzoic acid. This concentration of benzoic acid decreased the activity 60 per cent.

Substance	Inhibition, 10^{-4} M benzoic acid = 1
o-Aminobenzoic acid	0.25
m-Aminobenzoic "	0.33
o-Hydroxybenzoic acid	0.32
m-Hydroxybenzoic "	0.21
Terephthalic acid	0.19
2,4,5-Triiodobenzoic acid	0.13
p-Nitrobenzoic acid	0.80

component of the oxidase made from kidney extract to which benzoic acid was added had the same activity as preparations made from untreated extracts. (*c*) The protein component can be recovered without loss of activity from solutions containing benzoic acid by treatment (7) with acid and ammonium sulfate. (*d*) Flavin-dinucleotide precipitated as the silver salt (7) from solutions containing benzoic acid had the same activity, after regeneration with hydrogen sulfide, as that precipitated from pure aqueous solution.

Assuming the formation of a reversibly dissociating substrate-enzyme complex and inhibitor-enzyme complex as suggested by

Michaelis and Menten (8), the relation between the concentrations of the components of an oxidase solution containing amino acid and benzoic acid would be

$$\text{(I)} \qquad \frac{(\text{Amino acid})\ (\text{benzoic acid-oxidase})}{(\text{Benzoic acid})\ (\text{amino acid-oxidase})} = \frac{K_{\text{amino acid}}}{K_{\text{benzoic acid}}}$$

The parentheses indicate concentration and K the dissociation constants of the complexes.

TABLE II

Calculation of $K_{\text{amino acid}}/K_{\text{benzoic acid}}$ from Equation I

The reconstructed oxidase was used in Experiments 1, 2, 3, and 4. 3 mg. of protein preparation plus 2.4 γ of flavin-dinucleotide were used in Experiments 1 and 4, 3 mg. of protein plus 4.8 γ of flavin in Experiment 3, and 6 mg. of protein plus 2.4 γ of flavin in Experiment 2. The kidney extract was used in the other experiments. The oxygen uptakes given in Experiments 2, 3, and 4 are for 7.5 minutes; the others for 10 minutes.

Experiment No.	Concentration of d(−)-alanine	Concentration of benzoic acid	Oxygen uptake			$\frac{K_{\text{amino acid}}}{K_{\text{benzoic acid}}}$
			Without benzoic acid	With benzoic acid	Inhibition	
	M	M	microliters	microliters	microliters	
1	0.0028	0.0001	24	5	19	106
2	0.0056	0.0001	72	24	48	112
3	0.0056	0.0001	48	16	32	112
4	0.0056	0.0001	44	14	30	120
5	0.0560	0.0001	45	36	9	140
6	0.0280	0.0001	35	24	11	128
7	0.0056	0.0001	20	8	12	84
8	0.0028	0.00001	21	14	7	140
9	0.0028	0.0001	21	5	16	90
10	0.0028	0.001	21	0.5	20.5	115
Mean						115

In Table II are given data which permit the calculation of the ratio indicated in Equation I and the values of the ratio obtained. The rate of oxygen uptake in the absence of benzoic acid was taken as a measure of the concentration of total oxidase. The rate in the presence of benzoic acid was taken as a measure of (amino acid-oxidase). The difference between these rates was used as a measure of (benzoic acid-oxidase). It was assumed that the concentration of bound amino acid and benzoic acid was small compared to the total concentrations, and that the concentration of amino acid was the same in the absence and presence of benzoic acid. For convenience the initial concentration of *d*-amino acid was used.

Considering the variety of experimental conditions under which the data were obtained, the values for the ratio are in good agreement. Although the agreement cannot be considered as definite proof of the formation of a benzoic acid-oxidase complex, it does make plausible the assumption that the inhibitory effect is due to the formation of such a complex.

The light absorption of solutions of the reconstructed oxidase and of flavin-dinucleotide at wave-lengths of 350 to 500 mμ was not affected by the presence of 10^{-3} M benzoic acid. This suggests that benzoic acid does not combine with the flavin or protein component of the oxidase.

SUMMARY

1. The activity of the *d*-amino acid oxidase was decreased by benzoic acid; *e.g.*, the rate of oxidation of 1 mg. of *d*(−)-alanine by preparations of the oxidase was decreased about 60 per cent by 10^{-4} M benzoic acid.

2. Data were obtained which suggest that the inhibitory effect of benzoic acid may be due to the formation of a *benzoic acid-oxidase* complex.

Part of the expenses of this study was defrayed by a grant from the Research Council of Duke University.

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BIOCHIMICA ET BIOPHYSICA ACTA

BBA 65768

THE RELATION BETWEEN TEMPERATURE-INDUCIBLE ALLOSTERIC EFFECTS AND THE ACTIVATION ENERGIES OF AMINO-ACID OXIDASES

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(Received February 14th, 1968)

SUMMARY

1. In the catalytic reaction at 37° of D- and L-amino-acid oxidases with D-alanine and L-leucine, respectively, as substrate, benzoate and its derivatives act as competitive inhibitors. The Hammett plots show no straight-line relationship between the K_i and the σ values of the different substitutents.

2. With D-amino-acid oxidase (D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) at 10° in the $1/v$ vs. $1/[\text{D-alanine}]$ plot, the lines obtained in the presence and in the absence of inhibitor (benzoate or its derivatives, with the exception of the nitro-substituents), intercept at finite [D-alanine]. This was also the case with D-methionine as substrate. At 25° with D-methionine substrate inhibition is found at relatively high concentrations, which is not observed with D-alanine. This substrate inhibition can be abolished by benzoate and ATP, but not by *m*- or *p*-nitrobenzoate. ATP acts as non-competitive inhibitor, but only inhibits the reaction partially. At 37° the inhibition by ATP is abolished by benzoate. Titration of D-amino-acid oxidase with benzoate showed that, to obtain 50% saturation, 2-3 times more benzoate is necessary in the presence of an excess of ATP than in its absence. ATP itself does not induce spectral shifts.

It is concluded that two forms of D-amino-acid oxidase are present, a low-temperature and a high-temperature conformation, which have about the same activation energies, but differ in activity, due to different transition probabilities ($\Delta S^{\neq} = -0.7$ e.u.). ΔH for this conformational change is 55 000 cal·mole^{-1}; ΔS = 185 e.u. It can be shown that benzoate and its derivatives have more affinity for the high-temperature conformation, while ATP has more affinity for the low-temperature form; *m*- and *p*-nitrobenzoate have equal affinity for both forms.

3. With L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2) it was found that ATP and pyrophosphate influence the catalytic oxidation reaction. The linear Arrhenius plot obtained with L-leucine as substrate is converted by the addition of pyrophosphate into a Z-shaped curve. The substrate inhibition in the presence of L-leucine is abolished by pyrophosphate at low temperature. In the presence of L-valine as substrate a Z-shaped curve is obtained, of which, as in the case of L-leucine, the high-temperature and low-temperature parts are parallel. In the presence of pyrophosphate the curve shows one break. As in the case with D-amino-

acid oxidase, this enzyme also exists in two conformations with different activities and entropies of activation ($\Delta S^* = 1.8$–2.3 e.u.), but with the same activation energies. From the transition it can be calculated that $\Delta H = 46\,000$ cal·mole^{-1} and $\Delta S = 145$ e.u. for the conformational transition from the low- to the high-temperature form.

4. The general phenomenon of non-linear Arrhenius plots of enzyme-catalyzed reaction is discussed in connection with these observations.

INTRODUCTION

NEIMS, DELUCA AND HELLERMAN[1] investigated with D-amino-acid oxidase (D-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3) the relationship between the logarithm of the maximum velocity and the σ values for *m*- and *p*-substituted phenylalanine and *C*-phenylglycine. They obtained an asymmetrical, biphasic plot with a maximum at $\sigma = +0.04$ for *C*-phenylglycine and at $\sigma = +0.23$ for phenylalanine. Although it is known that benzoate[2] and some substituted benzoates[3] are competitive inhibitors of this enzyme, no attempt was made to investigate a correlation between the inhibition constants and the corresponding σ values.

With L-amino-acid oxidase (L-amino-acid:O_2 oxidoreductase (deaminating), EC 1.4.3.2), RADDA[4] investigated the correlation between the velocity and the σ value of ring-substituted phenylglycines as substrates in one concentration. As with D-amino-acid oxidase, benzoate is a competitive inhibitor for L-amino-acid oxidase[5]. ZELLER AND CLAUS[6] reported, however, that they could not find a correlation between the v_{max}, K_m and K_i determined for ring-substituted phenylalanine as substrate, the various benzoic acids as inhibitors and the σ values.

MASSEY, CURTI AND GANTHER[7] reported that D-amino-acid oxidase shows a discontinuous Arrhenius plot, from which it was concluded that the enzyme exists in two forms, a low-temperature form and a high-temperature form, in equilibrium with each other, having different activation energies. The temperature at which the break in the plot occurs, is dependent on the substrate used. With D-alanine it is at about 14°, while with D-methionine it is at 24°. In connection with fluorescence and ultraviolet-spectral studies, it was concluded that D-alanine has equal affinities for both forms, while D-methionine has more affinity for the low-temperature form than for the high-temperature form. With other enzymes a different explanation has been given for this phenomenon, *e.g.* an equilibrium between two or more forms differing in their catalytic activities[8] or a temperature-induced conformational change of the active site[9].

In this paper we present evidence that benzoate, substituted benzoates and ATP have different affinities for both forms, which differ in catalytic activity. Furthermore it will be shown that L-amino-acid oxidase can undergo temperature-dependent conformational changes, which depend on the substrate used and are influenced by ATP and by pyrophosphate.

Preliminary data of this work have been published elsewhere[10,11].

METHODS

D-Amino-acid oxidase (EC 1.4.3.3) was prepared from hog kidneys according to the method of MASSEY, PALMER AND BENNETT[12] and was made benzoate free by treatment with excess of D-alanine[13]. L-Amino acid oxidase (EC 1.4.3.2) was isolated

from snake venom (*Crotalus adamanteus*) according to the method of WELLNER AND MEISTER[14].

The enzymatic activities were measured with the Gilson Oxygraph Model KM. A polarizing voltage of −0.63 V was applied to the electrode. The oscillation frequency of the electrode was 120 cycles/sec and the greatest amplitude was used. The amount of O_2 consumed was calculated from the slope of the recorded line. The conditions described by BURTON[2], excepting catalase were used for D-amino-acid oxidase; for L-amino-acid oxidase, those described by WELLNER AND MEISTER[14] were used.

Agla micrometer syringes (Burroughs Wellcome and Co., London) were used for the spectrophotometric titration experiments. The optical differences were recorded with the 0–0.1 absorbance slide wire of the Cary model 14 recording spectrophotometer. The differences were recorded at 497.5 nm.

MATERIALS

D-Alanine and D-methionine were obtained from Fluka, Switzerland; ATP and GTP from Boehringer and Söhne; L-leucine, benzoate and the substituted benzoates from British Drug Houses. L-Leucine was recrystallized; some of substituted benzoates (B.D.H.) were first treated with charcoal after which they were recrystallized.

RESULTS AND DISCUSSION

The relation between the inhibition constants and the σ values of the substituted benzoates

Benzoate and the substituted benzoates inhibit D-amino-acid oxidase competitively. Table I summarizes the inhibition constants of benzoate and the ring-substituted benzoate derivatives for D-amino-acid oxidase, measured at 37° with D-alanine as substrate. In general the *o*-substituted benzoates give a lower inhibition than the *m*- and *p*-substituted derivatives, the *m*-substituents giving the highest inhibition. Exceptions are the hydroxy- and amino-substituents, of which the *o*-

TABLE I

THE INHIBITION CONSTANTS (μM) OF BENZOATE AND BENZOATE DERIVATIVES IN THE CATALYTIC OVERALL REACTION OF D-AMINO-ACID OXIDASE

The inhibition constants were determined by means of the Lineweaver–Burk plots. These experiments were performed at 37° in 0.1 M pyrophosphate buffer pH 8.30 with D-alanine as substrate.

Substituent	K_i (μM) *of*		
	o-	*p*-	*m*-
H	20.4		
NO_2	1370	38	11.1
OCH_3	1130	110	208
OH	76	330	251
F	294	3.2	2.9
Cl	361	10.6	2.9
Br	385	10.2	2.9
I	648	20.5	12.6
$N(CH_3)_2$		1000	1000
NH_2	103	313	794

[illegible]

substituted derivatives give the highest inhibition, probably due to the formation of hydrogen bridges. The lower inhibition with the other *o*-substituted benzoates could be due to steric hindrance, but *o*-fluorobenzoate (fluoro is in size comparable with hydrogen) has an inhibition constant of the same order as the other halogen-substituted benzoates. The results confirm those of BARLETT[15] and the spectrophotometric experiments of MASSEY AND GANTHER[16].

Fig. 1 shows the logarithm of the inhibition constants of the benzoate-derivatives for D-amino-acid oxidase obtained at 37°, plotted against the σ value. The line drawn

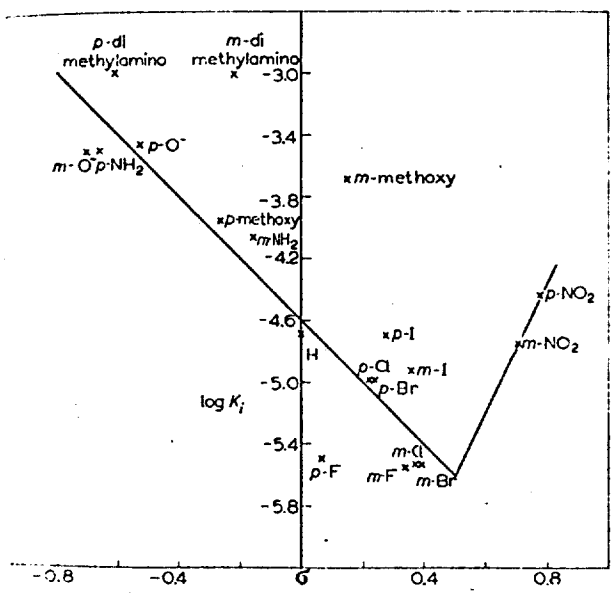


Fig. 1. Hammett plot of the action of ring-substituted benzoate derivatives with D-amino-acid oxidase. The logarithms of the inhibition constants at 37° are plotted against their σ-values. The K_i's were determined from Lineweaver-Burk plots; D-alanine was used as substrate. The line drawn was calculated by the method of the least squares; in this calculation *m*-dimethylaminobenzoate and *m*-methoxybenzoate were not taken into account for reasons of steric hindrance.

calculated by the method of the least squares. The σ value is experimentally obtained for the individual substituents[17]; the σ value of hydrogen is arbitrarily chosen as zero, and a positive value of σ means a stronger electron attracting character than hydrogen. The plot is biphasic with a minimum at $\sigma = -0.5$. The slope of the line of the substituents with a σ value lower than 0.5 has a ϱ value of -2.0, with a correlation coefficient $r = -0.6$.

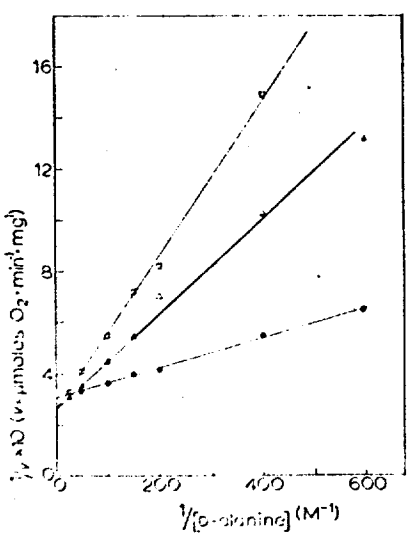
From the plot it can be concluded that the inhibition of the catalytic reaction increases with increasing electron-attracting character (increasing σ value) of the substituents. The other part of the plot is formed by the *m*- and *p*-nitrobenzoates. The slope of this part has a ϱ value of about $+4.0$. The substituents with a σ value smaller than 0.5 have a $-I$ and $+R$ effect, while the nitro derivatives have a $-I$ and $-R$ effect. NEIMS, DELUCA AND HELLERMAN[1], working with ring substituted substrates, found that increasing σ values lead to an increasing maximum velocity up to

a σ value depending on the substrate used. At a higher σ value, a decline in activity is observed.

A similar plot made for L-amino-acid oxidase differs in some respects from that of D-amino-acid oxidase, but the behavior of *m*- and *p*-nitrobenzoate is similar to both enzymes. Other substituted benzoates with negative σ values have approximately the same inhibition constants with L-amino-acid oxidase. The *m*-halogen-substituted derivatives, on the other hand, show a sharp increase in inhibition. This behavior at 37° differs only quantitatively from that at 25°; binding of these derivatives induce a shift of the flavin absorption band to shorter wavelengths[18]. The results show that there is a correlation between σ value and the inhibition constant in agreement with the results of RADDA[4] and in contrast to the results of ZELLER and co-workers[5,6].

The effect of benzoate, substituted benzoates and ATP on D-amino-acid oxidase

The $1/v$ vs. $1/[\text{D-alanine}]$ plot at 10° of Fig. 2 shows that the line obtained in the presence of benzoate (similar phenomena are found with substituted benzoates) intersects the line obtained in the absence of inhibitor at finite concentration of D-alanine. At temperatures of 15° and higher benzoate and derivatives are competitive inhibitors (*cf.* ref. 19); on the other hand, *m*- and *p*-nitrobenzoate exhibit strictly competitive behavior over the temperature range 5–40°. The same phenomenon is evident at 10° with D-methionine as substrate (Fig. 3). In this case the effect disappears as the temperature is raised above 24° and the inhibition becomes competitive. The most striking phenomenon in these experiments is the observation that, at high concentration of both D-alanine and D-methionine, the inhibitory effect of benzoate is replaced by an activating effect. In this respect benzoate and its derivatives behave like allo-



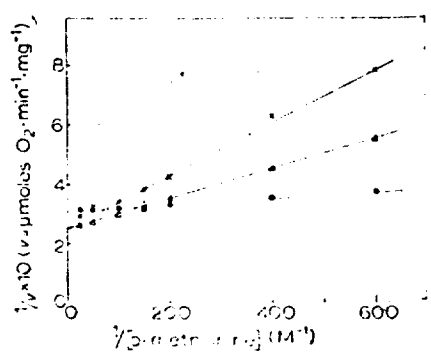


Fig. 2. Effect of benzoate on D-amino-acid oxidase with D-alanine as substrate. Conditions described under METHODS. Temp., 10°. O—O, no benzoate; [illegible], benzoate [illegible] 5 μM; [illegible] benzoate [illegible] 10 μM.

Fig. 3. Effect of benzoate on D-amino-acid oxidase with D-methionine as substrate. Conditions described under METHODS. Temp., 10°. O—O, control; [illegible], benzoate [illegible] 7.5 μM; [illegible] benzoate [illegible] 15 μM.

steric inhibitors (*cf.* refs. 20, 21), with the difference that the activating effect is not visible at low substrate concentrations. The model of MONOD, WYMAN AND CHANGEUX[21], which approaches this phenomenon mathematically, predicts that a concerted transition mechanism will show substrate inhibition in case the substrate has a higher affinity to a catalytically inactive than to a catalytically active state.

If the discontinuity of the Arrhenius plot[7] is due to the presence of an inactive form at lower temperature, it can be expected that substrate inhibition will be observed in case the substrate has a higher affinity for the inactive form. When the low temperature form is less active rather than inactive, as assumed in the allosteric theory, only under certain conditions can substrate inhibition be observed. By assuming that the enzyme concentration is much smaller than the substrate concentration, one can derive that, for the steady-state approximation, the general equation for the initial rate of the reaction becomes:

$$v = \frac{n\{(1+\alpha)^n v^R_{max}[S]/([S]+K_m^R) + L(1+c\alpha)^n v^T_{max}[S]/([S]+K_m^T)\}}{(1+\alpha)^n + L(1+c\alpha)^n} \qquad (1)$$

n is the number of substrate binding sites; L is the equilibrium constant between the R-form and T-form free of ligand, defined as $[T] = L[R]$; α is the ratio of the free substrate concentration and the dissociation constant for the binding to the R-form; c is the ratio of the dissociation constants for the R- and the T-form, *i.e.* $c = K^R_D/K^T_D$; v^T_{max} and v^R_{max} are the maximum velocities for the T-state and R-state, respectively, per catalytic site; K^T_m and K^R_m are the Michaelis constants for the T-state and the R-state, respectively. It must be pointed out that v^T_{max}, v^R_{max}, K^T_m and K^R_m vary with the concentration of the second substrate[19]. Furthermore it is assumed that this second substrate does not influence the equilibrium between R and T. This equation which is derived from the fraction of protein in the R- and T-states is valid, provided the

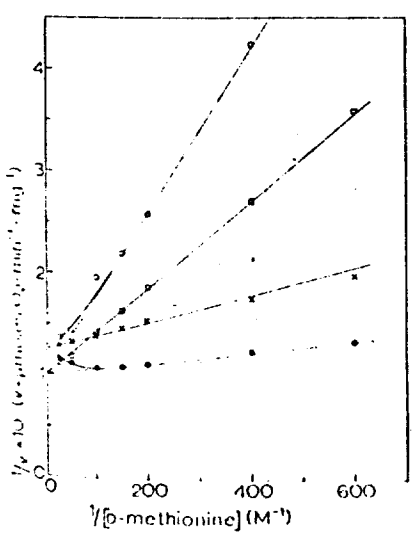


FIG. 4. Effects of effectors on the substrate inhibition of D-amino acid oxidase with D-methionine as substrate. Conditions described under METHODS. [illegible] D-methionine plot with different additions at 25°. O O, control; [ ] [ ], benzoate [illegible] 75 μM; [illegible] nitrobenzoate [illegible] mM; × ×, [ATP] = 20 mM.

equilibrium between these forms is established at the time of the first rate measurement. This equation is, in our opinion, preferable to the relation of the ligand saturation function with the velocity (*cf.* ref. 22), since this binding function does not take into consideration other possible intermediates in the conversion of substrate to product. The rate is determined by the normal kinetic parameters. Since the K_m for most enzymes is not related to the dissociation constant of the enzyme substrate complex, it is better not to use the latter in these equations unless a special mechanism is involved (*cf.* refs. 23, 24).

It can be seen from Eqn. 1 that in the case when the equilibrium constant L is small (but the substrate has more affinity for the less active T-form) at high concentration substrate inhibition will be observed. Fig. 4 shows that this is the case at high concentration of D-methionine. At higher or lower temperatures, this inhibition disappears. With D-alanine no substrate inhibition is observed at any temperature, indicating that this substrate has about equal affinities for both forms (*cf.* ref. 7). The observation (Figs. 2 and 3) that the activating effect of benzoate with D-methionine is more pronounced than with D-alanine is in agreement with the above-mentioned conclusion, provided benzoate has a higher affinity for the R-conformation. Furthermore these figures show that the plots in the presence of inhibitor remain straight at lower concentrations of benzoate. At higher levels of benzoate, deviation from linearity is observed at high concentrations of D-methionine. Extrapolation of the initial slope to infinite substrate concentration gives the same interception on the $1/v$ axis as obtained with the line at low level of benzoate. These results indicate that benzoate has a higher affinity for the high-temperature form than for the low-temperature state.

The presence of a non-reacting ligand binding to both states with different affinities modifies Eqn. 1 into:

$$v = \frac{n\{(1-\alpha)^n(1-\beta)^n v^{R}_{max} S_o/(S_o + K_m^{R}) + L(1-c\alpha)^n(1-d\beta)^n v^{T}_{max} S_o/(S_o + K_m^{T})\}}{(1-\alpha)^n(1-\beta)^n + L(1-c\alpha)^n(1-d\beta)^n} \quad (2)$$

In case the ligand is a competitive inhibitor Eqn. 2 changes to:

$$v = \frac{n\{(1-\alpha-\beta)^n v^{R}_{max} S_o/(S_o + K_m^{R}(1+\beta)) + L(1-c\alpha-d\beta)^n v^{T}_{max} S_o/(S_o + K_m^{T}(1+d\beta))\}}{(1-\alpha-\beta)^n + L(1-c\alpha-d\beta)^n} \quad (3)$$

In these equations β is the ratio of the free-ligand concentration and the dissociation constant of the R-form; d is the ratio of the dissociation constants of the binding of the ligand to the R- and T-form.

The main factors which determine the effects to be observed are L, c and d. Thus it can be expected that the substrate inhibition observed without benzoate (Fig. 4) is abolished upon benzoate addition. At the temperature used in these experiments, L is small and $c > 1$, which exerts its influence only at very high D-methionine concentrations. By adding benzoate $d < 1$, this effect is delayed and substrate inhibition is apparently abolished. The results obtained are those for true competitive inhibition.

Nitrobenzoates (*m*- and *p*-) act as true competitive inhibitors over the temperature range 5–40° with D-alanine as substrate. With D-methionine as substrate, however, although extrapolation on the initial slope indicates a true competitive inhibition (Fig. 4), at high concentrations of substrate deviation from the linearity and substrate inhibition occurs. It must be pointed out, however, that this only occurs

at about 25°. At higher or lower temperatures the inhibition is competitive. In connection with the ideas developed, it is clear that these inhibitors have about equal affinities for both forms ($d \approx 1$).

With D-alanine as substrate ATP is a non-competitive inhibitor. The concentration of ATP which inhibits the activity is strongly temperature dependent, for instance at 5° hardly any inhibition is observed in the presence of 200 μM ATP and a small inhibition (less than 8%) with 20 mM ATP; at 37° 300 μM ATP gives an inhibition of 26% (Figs. 5A, B). Furthermore it must be remarked that GTP in about the same concentration gives similar results. Fig. 4 shows that, in the presence of a large concentration of ATP, no substrate inhibition is obtained with high [D-methionine]. ATP inhibits the enzyme only partially, since a 100-fold increase in concentration does not affect the inhibition very much (Fig. 6). Furthermore at that high ATP concentration, in contrast to the low, substrate inhibition is observed. Although only at very high concentration of D-methionine is a deviation from the linearity observed, it must be remarked that three different experiments show this effect.

These results indicate that ATP has a higher affinity for the low-temperature conformation than for the high-temperature form. This non-competitive inhibition is due to a shift of the equilibrium between the two states. This equilibrium lies at 37°

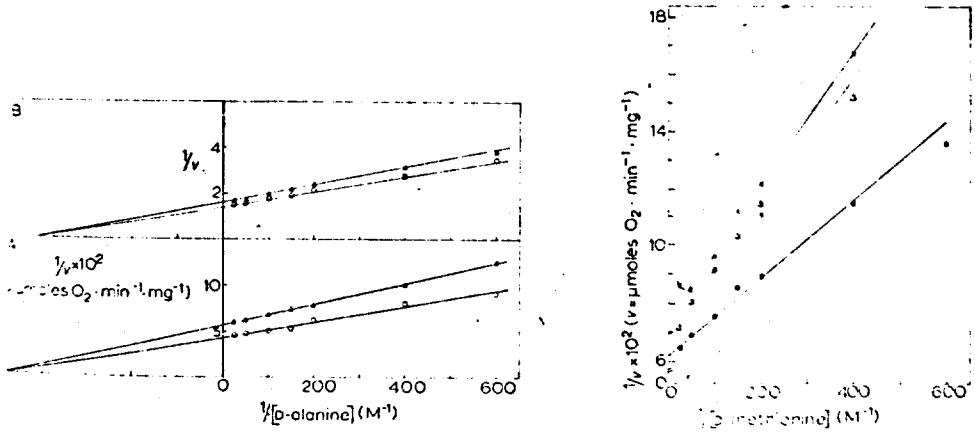
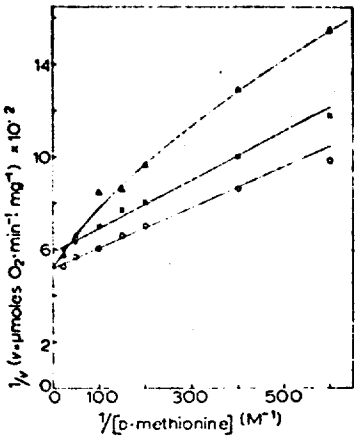


Fig. 5. Effect of temperature on the inhibition of D-amino-acid oxidase by ATP with D-alanine as substrate. Conditions described under METHODS. A. Temp., 37°, ○—○, control; ●—●, ATP [illegible] μM. B. Temp., 5°, ○—○, control; △—△, ATP [illegible] 20 mM.

Fig. 6. Effect of ATP on D-amino-acid oxidase with D-methionine as substrate at 37°. Conditions described under METHODS. ○—○, control; △—△, ATP [illegible] 200 μM; [illegible] ATP [illegible] 20 mM.

toward the R-form, at 5° toward the T-form. It is the cooperation between D-methionine and ATP, both having higher affinity to the T form, which at 37°, where the allosteric constant is small, causes substrate inhibition only at very high concentrations of both compounds.

Figs. 7 and 8 provide further evidence for the idea that benzoate and ATP have their highest affinity to different forms of the enzyme. Fig. 7 shows that the non-competitive inhibition of ATP at 37° is abolished by the addition of benzoate. The fact that the curve in the presence of benzoate is convex towards the abscissa is in



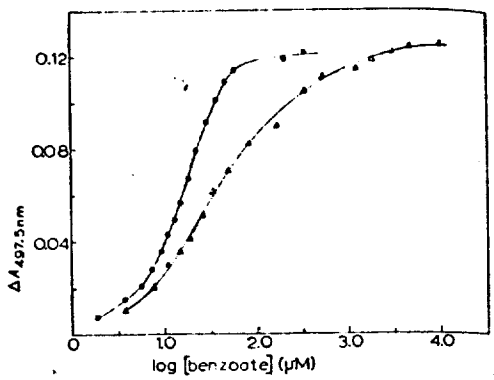


Fig. 7. Effect of ATP and benzoate on D-amino-acid oxidase with D-methionine as substrate at 37°. Conditions described under METHODS. ○—○, control; △—△, [ATP] = 200 μM + 10 μM benzoate; ×—×, [ATP] = 200 μM.

Fig. 8. Effect of ATP on the formation of the spectral enzyme benzoate complex of D-amino-acid oxidase. Differences in $A_{497.5\ nm}$ obtained by titration with benzoate are plotted. The values are corrected for dilution. ○—○ without ATP; △—△, in the presence of 20 mM ATP. Temp., 4.9°. [Enzyme-flavin] = 32 μM in 0.1 M pyrophosphate buffer (pH 8.3).

agreement with the ideas outlined here. At low concentrations of D-methionine the activating effect of benzoate is larger than at higher concentrations, since it can more easily overcome the synergistic action between ATP and the substrate.

The binding of benzoate induces a red shift in the flavin absorption with a maximum at 497.5 nm (refs. 16, 25–27). ATP itself does not induce a spectral shift. When the enzyme is titrated with benzoate a 2–3-fold higher concentration of benzoate is needed for 50% saturation in the presence of ATP (Fig. 8). The non-competitive inhibition of ATP at 37° and the small inhibition at 5° indicates that the counteraction by ATP of the benzoate binding is not due to binding of the nucleotide to the active site. The titration curves do not show signs for cooperative interactions. On the other hand, the curve in the presence of ATP is very asymmetrical; that in the absence of ATP is not completely symmetrical. MONOD, WYMAN AND CHANGEUX[21] have pointed out that the S-shape of the titration curves is dependent on the value of the allosteric constant L and the ratio of the dissociation constants c. The fact that, in the presence of 20 mM ATP, no S-shaped curve is observed is due to the binding of benzoate to both forms, provided that benzoate also gives the same spectral complex with the low-temperature form.

Further evidence for the presence of two conformations with different velocities and affinities comes from the following experiments. The Arrhenius plot (Fig. 9) of the maximum velocity with D-methionine shows a break point at 24° (*cf.* ref. 7). In the presence of benzoate however, the break becomes less sharp, when the extrapolation of the linear part of the Lineweaver–Burk plot is taken as maximum velocity (*cf.* Figs. 3–5). In the presence of ATP, a fairly good straight line relationship is obtained, but with lower activities at temperatures higher than 5°. The plot in the presence of ATP (ΔH = 9900 cal·mole⁻¹) is almost parallel with the upper part of

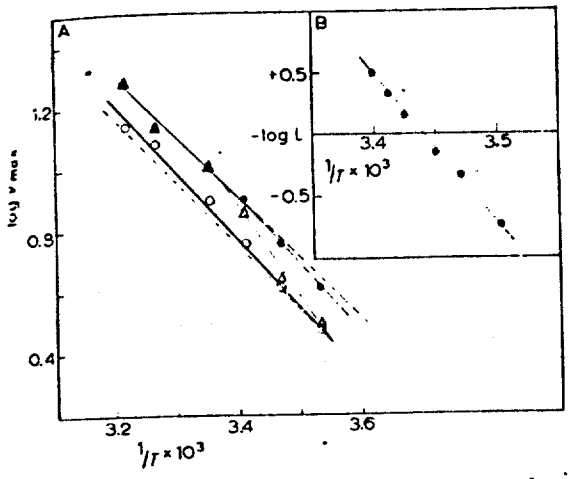


Fig. 9. A. Effect of effectors on the Arrhenius plot of D-amino-acid oxidase. The log v_{max} (μmoles $O_2 \cdot min^{-1} \cdot mg^{-1}$) with D-methionine as substrate, obtained by extrapolating the linear part of the Lineweaver–Burk plot, is plotted against $1/T$. △—△, control; ●—●, in the presence of 25 μM benzoate; ○--○, in the presence of 20 mM ATP. B. Van 't Hoff plot of the temperature-dependent equilibrium between the low- and high-temperature forms of D-amino-acid oxidase. The equilibrium constant L was calculated as described in the text.

the Arrhenius plot without any effector present (ΔE = 9300 cal·mole^{-1}). The fact that the plots are not completely parallel is partly due to the fact that we were working at rather low ATP concentrations to avoid substrate inhibition (*cf.* Fig. 6). This gives a small increase in maximum velocity due to the presence of some R-form, which results in a small increase of the activation energy. A close examination of the experimental points reveals that the tendency of a break is still present. Affinity for both conformations in the case of benzoate binding leads to the Arrhenius plot not being completely straight. The presence of the T-form with lower activity leads to a smaller value of the maximum velocity. Therefore it is concluded that the two forms have the same activation energy but differ in maximum rate, indicating that identical transition states have different probabilities. From the difference in rates it can be calculated that the difference in entropy of activation between the R- and T-forms ΔS^{*} = 0.7 e.u.

If for a certain condition v_{max} is the experimentally obtained maximum velocity, v^{T}_{max} the maximum velocity of the T-form, v^{R}_{max} the maximum velocity of the R-form, f_R the molar fraction of the R-form, and $1 - f_R$ the molar fraction of the T-form, then for the equilibrium between the two forms the following relations are valid:

$$v_{max} = f_R v^{R}_{max} + (1 - f_R) v^{T}_{max} \qquad f_R = \frac{v_{max} - v^{T}_{max}}{v^{R}_{max} - v^{T}_{max}}$$

and

$$L = \frac{[T]}{[R]} = \frac{1 - f_R}{f_R}$$

Since the activation energies are the same, the f_R value can be calculated for the temperature traject where the transition occurs and connected with this the allosteric

constant L. From the Van 't Hoff plot of the temperature-dependent variation of L (Fig. 9B), it can be calculated that $\Delta H = 55\,000$ cal·mole^{-1} and $\Delta S = 185$ e.u..

It is rather difficult to explain the kinetic results with the temperature-dependent association[28] observed with this enzyme. In that case the high-temperature form is the polymer of the low-temperature form. The results indicate that benzoate has a higher affinity for the R-form, but it has been reported that in the presence of benzoate the enzyme is less able to polymerize (*cf.* ref. 28). It is thus more likely that the results obtained are more due to conformational changes of the protein rather than to polymerization.

Temperature-dependent conformational changes of L-*amino-acid oxidase and the effect of pyrophosphate and ATP*

Comparison of the kinetic properties of L-amino-acid oxidase with those of D-amino-acid oxidase (*cf.* ref. 29) indicates the possibility that the large substrate inhibition observed with L-leucine as substrate and the fact that hardly any substrate inhibition is found with L-valine (*cf.* ref. 30) might be due to an equilibrium between two forms of the enzyme. Similarly these two forms must have different maximum rates and different affinities for the substrate. From the large inhibition with L-leucine, it could thus be concluded that this substrate must have a higher affinity for the less active low-temperature form; therefore L-valine must have about equal affinity for both forms.

The application of the results obtained with D-amino-acid oxidase to the Arrhenius plots of L-amino-acid oxidase provides evidence that the latter enzyme also exists in two temperature-dependent conformations with different activities.

In Fig. 10 where the Arrhenius plot with L-leucine as substrate is presented, a straight line is obtained. In the presence of 6.7 mM pyrophosphate (Fig. 11A) the Arrhenius plot changes considerably; the plot becomes Z-shaped by showing two

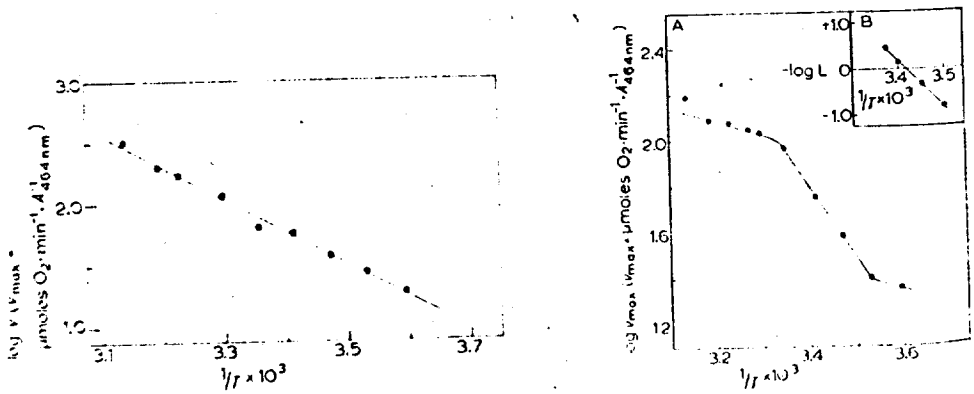


Fig. 10. The Arrhenius plot of L-amino-acid oxidase with L-leucine as substrate in 0.05 M Tris-KCl buffer (pH 7.5). Conditions described under METHODS. v_{max} obtained as described in Fig. 9A.

Fig. 11. A. Effect of pyrophosphate on the Arrhenius plot of L-amino-acid oxidase with L-leucine as substrate. Conditions the same as Fig. 10 but in the presence of 6.7 mM pyrophosphate (pH 7.5). v_{max} was obtained as described in Fig. 9A. B. Van 't Hoff plot of the temperature-dependent equilibrium between the two forms of L-amino-acid oxidase in the presence of 6.7 mM pyrophosphate. The equilibrium constant L was calculated as described in the text.

breaks at 26° and 10°. The high-temperature part is parallel to the low-temperature part, the three points at the lowest temperatures being averages of two independent determinations. From this plot it can be concluded that L-amino-acid oxidase exists in two temperature-dependent forms. The appearance of two breaks indicates that pyrophosphate shifts the equilibrium between the two forms towards the high-temperature form. Furthermore, it is clear that L-leucine must have a high affinity to the less active form, the conclusion strengthened by the fact that substrate inhibition occurs over the whole temperature range (5–45°). On the other hand, at low temperature in the presence of pyrophosphate there is practically no substrate inhibition. The activation energy calculated from the plot of Fig. 10 is about the same as the activation energy calculated form the transition range of Fig. 11A in the presence of pyrophosphate (Table II).

This indicates that the linear plot in the absence of pyrophosphate represents the transition traject. These results are a warning against the conclusion that a linear Arrhenius plot is the reflection of one enzyme conformation.

With D-amino-acid oxidase the Arrhenius plot was dependent on the substrate used; the same is the case with L-amino-acid oxidase. With L-valine as substrate, in

TABLE II

THE ACTIVATION ENERGIES ΔE CALCULATED FROM THE ARRHENIUS PLOTS OF THE CATALYTIC ACTION OF L-AMINO-ACID OXIDASE WITH L-LEUCINE AND L-VALINE AS SUBSTRATES AND THE INFLUENCE OF ATP (20 mM) AND PYROPHOSPHATE (6.7 mM)

Substrate	*Addition*	ΔE *(cal · mole⁻¹)*	*Temp. range (°C)*
L-Leucine	No	13 300	5–45
L-Leucine	Pyrophosphate	2 760	>26
		13 800	10–26
		2 760	<10
L-Valine	No	2 300	>40
		10 600	20–40
		2 300	<20
L-Valine	Pyrophosphate	2 300	>33
		11 500	<33
L-Valine	ATP	2 300	>40
		10 900	20–40
		2 300	<20

contrast with L-leucine, a discontinuous plot with two breaks (at 40° and 20°) is obtained (Fig. 12A). In the presence of 6.7 mM pyrophosphate just one break occurs at 33°, the second one at low temperature has disappeared. Again the shift of the break towards a lower temperature is an indication that pyrophosphate shifts the equilibrium towards the high-temperature form (*cf.* L-leucine with 6.7 mM pyrophosphate).

With D-amino-acid oxidase ATP has a higher affinity to the low temperature form; in the case of L-amino-acid oxidase ATP shifts the equilibrium slightly to the high temperature form with little influence on the temperature of the break (Fig. 13A).

Table II summarizes the values of the activation energies for the different temperature ranges with L-leucine and L-valine as substrates in the presence and absence of pyrophosphate and ATP. The enzyme used in the experiments of Figs. 12A and 13A are from different preparations; every experimental point is the average of two independent determinations.

In the same way as has been done with D-amino-acid oxidase, the enthalpy and entropy changes of the conformational transition can be calculated from Figs.

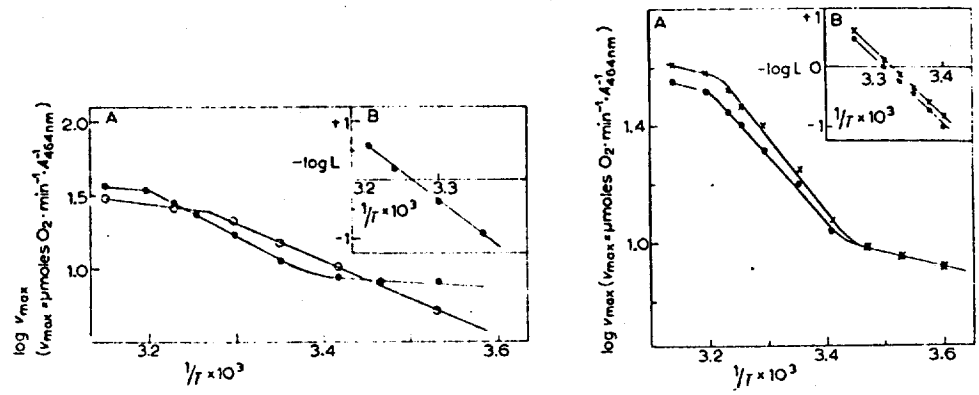


Fig. 12. A. The Arrhenius plot of L-amino-acid oxidase with L-valine as substrate in the absence and presence of pyrophosphate. Conditions as in Fig. 10. ●—●, no pyrophosphate; ○--○, with 6.7 mM pyrophosphate (pH 7.4). v_{max} was obtained as described in Fig. 9A. B. Van 't Hoff plot of the temperature-dependent equilibrium between the two forms of L-amino-acid oxidase. The equilibrium constant L was calculated as described in the text.

Fig. 13. A. Effect of ATP on the Arrhenius plot of L-amino-acid oxidase with L-valine as substrate. Conditions as in Fig. 10. ●—●, no ATP; ×--×, with 20 mM ATP. v_{max} was obtained as described in Fig. 9A. B. Van 't Hoff plots of the temperature dependent equilibrium between the two forms of L-amino-acid oxidase in the presence and absence of ATP. The equilibrium constant was calculated as described in the text. ●—●, no ATP; ×--×, ATP, 20 mM.

11B, 12B and 13B. For L-leucine in the presence of 6.7 mM pyrophosphate the value is 46 000 cal·mole^{-1}; L-valine alone 47 000 cal·mole^{-1} and L-valine with 20 mM ATP 46 000 cal·mole^{-1}; ΔS varies from 140–150 e.u.. From these values it seems that the ΔH is rather independent of the substrate and additions made, indicating that the differences between the two protein conformations are the main factor involved. Another conclusion is that conformational changes which occur upon substrate binding induce about the same changes in thermodynamic parameters of both the low- and high-temperature forms.

From the differences in rates the difference in entropy of activation for both forms under each condition is calculated; $\Delta S^{\ddagger}$ = 2.3 e.u. for L-leucine with 6.7 mM pyrophosphate; $\Delta S^{\ddagger}$ = 1.8 e.u. for L-valine and for L-valine with 20 mM ATP $\Delta S^{\ddagger}$ = 2.1 e.u.

The great difference between the two amino acid oxidases is the substrate inhibition, occurring with L-amino acid oxidase. In the literature different explanations are given for this inhibition[29,30]. It was assumed by WELLNER AND MEISTER[30] that the half reduced enzyme is reoxidized faster than the total reduced enzyme. This explanation was ruled out by the experiments of MASSEY AND CURTI[29]. They proved

that the reoxidation of the total reduced enzyme cannot be the rate-limiting step in the catalytic overall reaction and showed that the reaction mechanism was quite similar to that of D-amino-acid oxidase (*cf.* ref. 19). To explain the inhibition they assumed the formation of a complex between the fully reduced enzyme and the substrate. This complex should be unreactive or at least react slowly with oxygen. Our explanation of the substrate inhibition is similar to the explanation of the substrate inhibition occurring with D-amino-acid oxidase with D-methionine as substrate. The results shown above indicate that the behavior of the two oxidases is basically the same in spite of the apparent differences.

The phenomenon of non-linear Arrhenius plots is well known in enzymology. MASSEY, CURTI AND GANTHER[7] have recently given a list of enzymes showing these effects. Many explanations have been given, the majority rejected on theoretical grounds (*cf.* refs. 8, 9). Recent explanations deal with the existence of different protein conformations, either each with similar activity, but differing in energy of activation[7,31], or with different activities[8,9].

Our results strongly support the latter idea. They show that a non-linear Arrhenius plot is to be expected in case the enzyme can undergo a reversible temperature-dependent transition between two conformations with the same activation energies (*e.g.*, the same active complex) but differing in the probabilities of the transitions. Such a mechanism can only be explained in terms of conformational changes around the active site of the enzyme (*cf.* ref. 9). The results show clearly that cooperative effects[21] are observable in such a case, which leads to the question whether similar phenomena occur in other so-called allosteric enzymes. This is especially important in relation to very complex enzymes like glutamate dehydrogenase. Although explanations have been given for the marked substrate inhibition by the assumption of multiple binding sites for NADH (ref. 32), the results presented here show that other explanations are possible (*cf.* ref. 33). Furthermore, it must be considered that in two-substrate reactions, in the absence or presence of modifiers, all reagents can have different affinities for two or more conformations. This leads to a role of one substrate as an allosteric effector on the kinetic parameters of the second substrate. Variation of the concentrations of both substrates can lead to apparent contradictions. All these phenomena will be dependent on the kinetic mechanism, *e.g.* the case of either a modified enzyme mechanism, an ordered or a random mechanism.

These and other studies[31] show the importance of varying the temperature in testing involvement of different conformations. Furthermore, the results show that it is possible to observe two breaks, which according to these hypotheses are to be expected. Even the observation of a linear Arrhenius plot does not allow the conclusion that one conformation is involved. The results also provide an explanation for the exchange in the Arrhenius plot upon the addition of effectors to the enzyme-catalyzed reaction[8,9].

From the data obtained it is clear that the slope in the Arrhenius plot does not always represent the activation energy of the reaction. For determining the activation energy, the largest possible temperature range should be taken, because by using a limited temperature range there is a possibility, even upon finding a straight line relationship, that one is dealing with a transition between two conformations. In the transition range the slope represents, in addition to the activation energy, the change

in activation entropy of the reaction due to the conversion of the low-temperature conformation into the high-temperature one, both having different activities. In fact it is possible to distinguish two cases:

(1) The high-temperature form has a higher activity than the low-temperature conformation; in this case the part of the Arrhenius plot connected with the transition will have a larger slope than the parts of the plot representing either the pure high-temperature or the pure low-temperature conformation. Examples are the data presented here.

(2) The high-temperature form has a lower activity than the low-temperature conformation; in this case the part of the Arrhenius plot connected with the transition will have a smaller slope than the parts of the plot representing either the pure low-temperature or pure high-temperature conformation. An example might be the results obtained with fumarase (*cf.* ref. 31).

Whether these ideas are applicable to those obtained with other enzymes is an open question. However, many of the interpretations in enzyme catalysis based on activation energies have become doubtful in the light of these results and this interpretation.

Although a more physical explanation has been given for cooperative binding phenomena[35] in terms of the relation between sequential changes and ligand binding, our interpretations are more related to the concerted transition model of MONOD, WYMAN AND CHANGEUX[21]. However, it must be emphasized that an involvement of the induced-fit mechanism can not be ruled out by these experiments.

ACKNOWLEDGEMENTS

We gratefully acknowledge the technical assistance of Miss M. I. DRAISMA and wish to thank K. VAN LOON for performing preliminary experiments. Part of these investigations was subsidized by the Netherlands Foundation for Chemical Research (S.O.N.) with the financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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ABNORMAL PATTERN IN TISSUE PHOSPHOLIPIDS AND POTASSIUM PRODUCED IN RATS BY DIETARY SODIUM BENZOATE. PROTECTIVE ACTION OF GLYCINE

BY

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(*Received for publication 19-5-1959*).

Sodium benzoate is one of the important growth inhibiting toxic compounds. The detoxication of benzoate by conjugation with glycine and with glycuronic acid is well established. It was demonstrated by WHITE (1) that growth inhibition produced in rats by oral administration of sodium benzoate is alleviated by glycine and by compounds metabolically related to this amino acid. No detoxication was noted when sulphur-containing amino acids were used. White concluded that the inhibition of the growth of the rat by certain dietary foreign compounds resulted from a deficiency in the normal metabolic substance or substances specifically concerned with combination with the growth-inhibitory substance to form a detoxication product. It is apparent that this general statement based on the observation of growth inhibition in animals does not explain various metabolic changes which may occur during the process of intoxication and which are offset by the treatment with glycine.

The present work is concerned with some aspects of lipid and electrolyte metabolism in rats receiving toxic benzoate diet and protected or not by the addition of glycine.

MATERIALS AND METHODS

Male Sprague-Dawley rats having a weight range from [illegible]

The basal diet was a grain diet of the following composition (2): ground yellow corn 68 %; linseed oil meal 13 %; powdered skim milk 15 %; alfalfa leaf meal 2 %; cod liver oil 1 %; iodized table salt 0.5 % and calcium phosphate 0.5 %. The addition of 5 gm of sodium benzoate or of 1 gm of glycine to each 100 gm of basal diet constituted " benzoate diet " and " glycine diet " respectively. When both compounds were added to the basal diet in above mentioned proportions, the diet was called " benzoate-glycine diet ". The following 4 groups of rats were given the experimental diets during a 3 week period: 1. basal diet (control); 2. benzoate diet; 3. glycine diet; 4. benzoate-glycine diet.

After 3 weeks the animals were killed and their body weight recorded. The livers were dissected for lipid study and the gastrocnemius muscle was removed for the determination of skeletal muscle electrolytes. Total cholesterol was determined following the method of ABELL *et al.* (3). For the study of lipid phosphate the samples were extracted with alcohol-ether and the analysis carried out by the method of ZILVERSMIT and DAVIS (4).

Baird Flame Photometer was used for the study of sodium and potassium extracted from the tissue (5). Chlorides were determined by titration (6).

RESULTS

The effect of various diets, used in this experiment, on body weight, is seen from the data in Table I. It may be noted that benzoate diet resulted in significant inhibition of growth. In WHITE's experiment (1), where the same concentration of benzoate in diet was used, as in this study, the body weight of rats remained practically stationary during

TABLE I

Effect of various diets on body weight (B.W.) *changes in rats*

Diet	No. of rats	Change in B.W. %	
		Mean	Range
Control diet	10	+ 23	+ 19 — + 26
Benzoate diet	15	— 25 %	— 21 — — 29
Glycine diet	13	+ 25 %	+ 21 — + 30
Benzoate + Glycine diet	15	+ 7 %	+ 5 — + 13

TABLE II

Liver lipids (mg 100 gm wet tissue) in rats after feeding with various diets during a 3 week period
Mean, Standard error of the Mean ( ) and Range

Diet	No. of rats	Total cholesterol	Phospholipids	C/P ratio
Control	10	275	2728	0.10
		10	116	0.007
		230–323	2130–3150	0.09–0.12
Sodium benzoate	15	264	1469 (1)	0.19 (1)
		16	56	0.01
		255–274	1340–1625	0.18–0.27
Glycine	13	269	2720	0.10
		4	79	0.005
		228–347	2112–3122	0.09–0.14
Sodium benzoate Glycine	15	259	2825	0.09
		7	99	0.01
		234–314	2088–3440	0.08–0.11

(1) Statistically significant.

TABLE III

Electrolytes of skeletal muscle (mEq 100 gm fat-free solids) in rats after feeding with various diets during a 3 week period
Mean, Standard error of the Mean ( ) and Range

Diet	No. of rats	Na	K	Cl
Control	10	11.5	43.9	11.0
		0.3	0.6	0.2
		10.3–12.8	41.4–46.8	9.9–12.1
Sodium benzoate	15	11.3	35.8 (1)	13.4
		0.2	0.4	0.4
		10.9–12.3	33.7–37.1	11.8–15.7
Glycine	13	11.8	43.1	10.9
		0.2	0.4	0.2
		10.0–13.5	40.4–44.7	9.6–13.0
Sodium benzoate Glycine	15	11.2	44.2	10.7
		0.4	0.7	0.3
		9.0–13.7	41.4–48.2	7.–13.2

(1) Statistically significant.

the period of 3 to 6 weeks. In our study reduction of body weight was observed in all rats fed benzoate diet. It may also be seen from Table I that the amount of glycine added to benzoate diet partially protected the rats against the reduction of body weight.

Table II summarized data on lipid study in the liver. It may be seen that total cholesterol content is not affected by the experimental diets. There is however significant reduction of liver phospholipids in the group of rats fed benzoate diet. High C/P ratio in this group is the result of this decrease of phospholipids. Normal values of phospholipids were found in all other groups of rats. Concentrations of electrolytes in the skeletal muscles are recorded in Table III. A significant difference between the groups is a low concentration of potassium in benzoate fed rats. Normal values of this ion are observed in other animals.

DISCUSSION

It results from this experiment that some abnormal changes in lipid and electrolyte metabolism occur in rats fed sodium benzoate in the basal diet, and that these changes may be offset by the addition of glycine to this diet. It is apparent that many metabolic alterations are associated with the inhibition of growth resulting from the dietary toxic compounds. This report illustrates only two possibilities of such a metabolic effect. Liver phospholipids, which are reduced in benzoate fed rats are known to be essential components of all cells. They are present in the mitochondria and other cytoplasmic granules where they are associated with ribonucleoproteins and important enzyme systems. Because they seem to be relatively unaffected by such conditions as starvation, they are called "constant elements" of the depot fat (7).

The synthesis of certain phospholipids occurs mainly in the liver and the supply of labile methyl groups is needed for this process. It results from this experiment that the synthesis or storage of phospholipids in the liver is decreased in the benzoate fed rats and is normal in the presence of benzoate-glycine diet. The possible metabolic mechanisms of the effect of glycine on phospholipid synthesis will not be discussed in detail in this short note.

Another abnormality found in the benzoate fed rats is a low potassium content of skeletal muscle.

It is known that potassium is essential for normal growth and for reconstruction of tissue proteins (8). The interdependance of potassium and nitrogen metabolism is well established. The values of muscle

potassium found in this study are similar to those observed in the potassium deficiency induced by feeding the rats a low potassium diet (9). It was also found by MUNTWYLER and GRIFFIN (8) that the combined low potassium and low protein diet does not affect tissue potassium, but the low potassium diet alone results in rapid depletion of muscle potassium. These authors believe that a known impairment of nitrogen anabolism in potassium deficiency is not due to poor protein intake. It seems therefore that the low muscle potassium in benzoate fed rats is not due to the protein starvation, if any, but results from a more complex abnormality of cellular electrolyte storage and transport, provoked by toxic compound but specifically alleviated by the presence of supplementary glycine in the diet.

The results of this experiment demonstrate that some important alteration in the metabolism of tissue lipids and electrolytes are associated with sodium benzoate intoxication of rats. These alterations are absent if glycine is added to sodium benzoate diet.

SUMMARY

Sodium benzoate included in rats' diet provoked significant inhibition of growth and alteration in metabolism of lipids and electrolytes. Low liver phospholipids and low potassium of skeletal muscle was found in all rats fed sodium benzoate diet. Glycine added to sodium benzoate diet protected rats against above mentioned toxic effects of sodium benzoate.

ACKNOWLEDGMENT

Technical assistance of Mrs. M. SOLONY is gratefully acknowledged.

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THE EFFECT OF PRESERVATIVES ON THE UTILIZATION OF CAROTENE

by Magdalene KRAMER and R. TARJÁN

In winter months a certain part of the need of the population for vitaminA is covered to a certain extent by the carotene content of some canned vegetables and fruits in whose preparation preservatives are used routinely.

As is known, preserving agents exert an inhibition on the activity of different enzymes and can therefore influence metabolic processes. It may therefore be assumed that they are not indifferent for the utilization of carotene. Judging from the specialized literature accessible to us, the action of preservatives on the utilization of carotene has not yet been investigated. As this question is of practical importance we intend to define the potential effect of preservatives allowed and used in Hungary on the utilization of carotene, after having studied the different natural dietetic factors (2,3) and the metal impurities in food. (4)

Experimental part

To solve this problem we carried out the following experiments :

a) Using doses of preservatives which did not influence the digestion of food and the growth of experimental animals and supplying defined quantities of carotene the vitamin A content was estimated in liver and kidneys and compared with the vitamin A stock of the control animals.

b) Using preservatives which influenced the utilization of carotene, the experiment was repeated with smaller doses and subsequently the effect of prolonged administration was observed.

c) In order to identify the mechanism of action, the amount of carotene excretion was estimated after administration of different preserving agents so as to evaluate the potential effect on carotene absorption and its extent.

I. In the first series of experiments 40 8 weeks old male Wistar rats were distributed among four groups of 10 animals each. The animals received as staple food the cereal mixture used in previous experiments supplemented with caseine containing only traces of carotene (5). We mixed 2.4% of formic acid with the dry food of the first group, 2.0% of sorbic acid with that of the second group, 1.5% of sodium benzoate with that of the third. The fourth group served as control.
Formic acid and sodium benzoate were added to the food as a 10% water solution. To dissolve sorbic acid we used KOH in such quantity as to bring the pH to 7. The food was offered to the animals mixed with an equal amount of water so that the final concentration of preservatives in fact reached only half of the value calculated for the dry substance. In that way, in the moist food, the formic acid and sorbic acid concentration was about 10 times the normally permitted quantity and about 5 times for the sodium benzoate. By gradual increase of additions the animals got used to the preserving agents. With administration of sodium benzoate, it was observed that the animals ate less of the food when it contained 3% of the substance and therefore lost weight. These concentrations therefore had to be reduced. After 4 weeks of the diet the food of the experimental and control animals was supplemented daily with paprika puree containing 45 mg. of carotene per animal while the

preservatives were continued. The manner of dosage is described in our previous paper (4). After 2 weeks one half of the animals and 2 weeks later the other half were killed by decapitation and the content of vitamin A estimated in liver and kidneys. The analysis was carried out according to the method described in our previous paper (2).

2. On the basis of the first experiment we continued to study the noxious influence of formic acid and sorbic acid on the metabolism of carotene in a second series of experiments. First, we checked whether these substances have an inhibiting effect also in weaker concentrations and then we studied their effect after longer -6 weeks - ingestion. This series of experiments was carried out with 5 groups of 20 male rats each. 1.2 and 2.4% of formic acid and 1.0 and 2.0% sorbic acid calculated for dry food were mixed to the food, the fifth group serving as control. As in the first experiment, after 4 weeks, paprika puree was added to the food. After 2 weeks of carotene ingestion, one half of the animals was decapitated and because in the first experiment the changes in vitamin A content of the kidneys were inconsistent, we simply estimated the vitamin A content of the liver. With the other half of the experimental animals we did the same after 6 weeks carotene administration. Here liver and intestines were also examined histologically.

3. Finally we performed the carotene-balance-test to find out if preservatives influence the absorption of carotene. For the balance test, 2.4% formic acid, 20% sorbic acid, or 1.5% Na benzoic acid was mixed for 4 weeks to the food. The experiment was carried out in the previously described manner (4) on 5 rats for each group contrasted with 5 controls.

RESULTS

The results are compiled in Table 1 for the first series of experiments

Table 1: The influence of preservatives on the vitamin A content of the liver in rats

Konservierungsmittel	[illegible]	[illegible]	[illegible]	[illegible]
	nach zweiwöchiger Carotinzufuhr			
Kontrolltiere	10.0 ± 0.2	[illegible]	[illegible]	26 ± 7.7
2.4 % Ameisensäure	[illegible]	[illegible]	[illegible]	18 ± 4.4
2.0 % Sorbinsäure	11.0 ± 0.18	[illegible]	[illegible]	38 ± 16.4
1.5 % benzoesaures Natrium	10.1 ± 0.86	[illegible]	[illegible]	[illegible] ± 11.3
	nach vierwöchiger Carotinzufuhr			
Kontrolltiere	10.8 ± 0.67	[illegible]	[illegible]	30
2.4 % Ameisensäure	10.2 ± 1.09	[illegible]	[illegible]	29
2.0 % Sorbinsäure	10.1 ± 1.29	[illegible]	[illegible]	32
1.5 % benzoesaures Natrium	10.1 ± 1.[illegible]	[illegible]	[illegible]	32

*** < 1 %

The results show that with ingestion of formic acid and sorbic acid the vitamin A content of the liver after 2 weeks was lower than in the controls - in the formic acid group the difference is significant - on the contrary the vitamin A content of the liver in the group which received Na benzoic acid was not different from the controls. After 4 weeks the vitamin A content of the liver did not show any significant difference in any of the groups compared with the controls. Here also we found the least vitamin A in the liver of the formic acid group and somewhat more than in the controls in the Na benzoic acid group. Because of large scatter of single data the change in vitamin A content of the kidneys could not be evaluated. All the same it was noted that the change of kidney vitamin A content does not run parallel with

that of the liver.

The results of the second series of experiments are shown in Table 2

Table 2 :The influence of preservatives on the vitamin A content in the liver of rats

Konservierungsmittelzusatz	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
Kontrolltiere	[illegible]	21.6	[illegible]
1.2 % Ameisensäure	[illegible]	[illegible]	[illegible]
2.4 % Ameisensäure	[illegible]	[illegible]	[illegible]
1.0 % Sorbinsäure	[illegible]	[illegible]	[illegible]
2.0 % Sorbinsäure	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]
Kontrolltiere	[illegible]	[illegible]	[illegible]
1.2 % Ameisensäure	[illegible]	[illegible]	[illegible]
2.4 % Ameisensäure	[illegible]	[illegible]	[illegible]
1.0 % Sorbinsäure	[illegible]	[illegible]	[illegible]
2.0 % Sorbinsäure	[illegible]	[illegible]	[illegible]

* P < 5 %
** P < 2 %
*** P < 1

Here can be noted that formic and sorbic acid, even in weaker concentration than in the first experiment have an unfavorable effect. Formic acid was more damaging than sorbic acid, as the liver vitamin A content of animals which received formic acid in concentration of 1.2 and 2.4% was considerably lower than in the controls. The reduction was less in the sorbic acid groups, however the difference was significant when the food contained 2.0% of sorbic acid. After 6 weeks' carotene ingestion the difference between the liver vitamin A content in the sorbic acid and the control groups was not noticeable while the formic acid groups showed, with both concentrations, a significant

reduction of the stored liver vitamin A.

The result of the carotene-balance can be seen from Fig. 1. According to this, the animals which received formic and sorbic acids excreted significantly more carotene than the controls after ingestion of 90 µg carotene in the form of paprika puree. No difference could be observed in the carotene excretion of controls and Na benzoate fed animals.

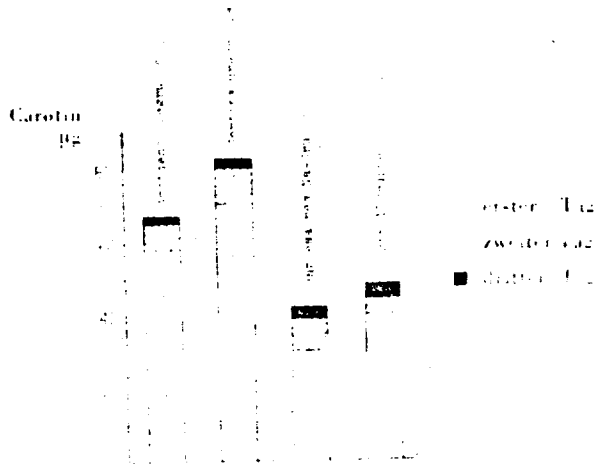


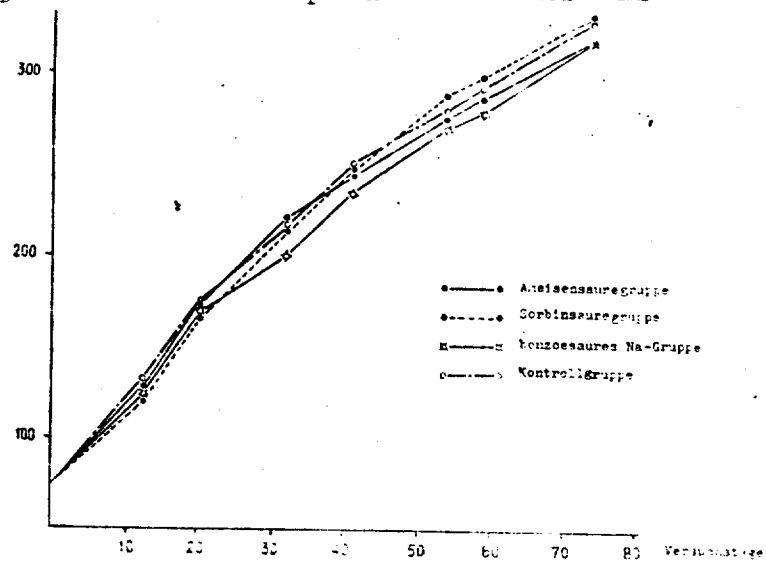
Fig.1 : Carotene content in feces of experimental animals on the first, second and third day after administration of 90 µg carotene

Fig. 2 shows the weight gain of each animal group. The weight curves are nearly equal with the exception of the animals which received Na benzoate. An inhibition appears here between the 20, and the 30, day. During that time the animals received 3% of Na benzoate in food that they did not consume completely. From the 32, day when the sodium benzoate in food was reduced to 1.5% the weight gain reached the level of the controls like the other 2 experimental groups.

Histological examination shows that the studied preservatives in the given concentrations (formic acid 2.4%, sorbic acid 2.0%, Na benzoate

1.5%) produce no discernable evidence of damage in the organs of rats after 10 weeks administration.

Fig 2 : Weight curves of experimental animals



DISCUSSION OF RESULTS

The aim of these experiments was to study the effects of preservatives in non-toxic quantities on the utilization of carotene. In the experiments we used concentrations which neither endangered the growth of animals, nor produced discernable histological changes in their organs.

According to the literature, 5% sorbic acid in food does not produce any inhibition of growth or reproduction after many months, even many years of administration; life is not shortened and no discernable histological changes can be observed. With an increase of the sorbic acid content in food to 8% DEUEL (7) noted an enlargement of the liver. The sorbic acid concentration of 1 and 2% used in our experiments did not influence the growth of animals nor did it cause any discernable histological changes in the organs of the experimental animals;

a certain enlargement of the liver could however be observed in comparison with the controls.

On the tolerance for Na benzoate opposite views are found in the literature. According to DEUEL (7) this substance at a concentration of 4% in the food and 90 days'administration does not cause any changes in the growth of animals or in weight of liver and kidneys , nor does it cause discernable histological changes in the organs. KIECHENBUSCH and LANG (8) , on the contrary, claim that a concentration of 1% of benzoic acid is already near the upper limit of tolerance. In their experiment a large part of the animals died within a few weeks after administration of 5% benzoate in food.

Our results confirm the observations of KIECKENBUSCH and collaborators as we found in contrast to DEUEL that rats ate less of the food which contained 3% of Na benzoate and lost weight after a few days while they showed no ill effect with a concentration of 1.5%.

WOEHLBIER and SIEBERT (9) gave to guinea pigs food which contained 4% formic acid and found that formic acid in that concentration was rapidly oxidized in the animal organism. The concentration of 1.2 and 2.4% used by us appeared harmless for growth as well as in regard to discernable histological changes of the organs.

On the basis of the vitamin A content of the liver, it can be concluded that formic and sorbic acids affect carotene utilization unfavorably while Na benzoate is without effect on it. Our results show that after 4 and 6 weeks'administration the stored vitamin A in liver deviates

less from that of the controls than after 2 weeks'administration. At that time the difference for the sorbic acid group is no longer significant. There is a possibility that the organism gets used to preservatives and becomes insensitive to the harmful effects after a while. In order to clarify the point, animal experiments of long duration must be undertaken.

In relation to the action mechanism we observed that formic and sorbic acids act as inhibitors of the absorption of carotene. As preservatives reduce enzymes activity as well as lipase (1.6) it can be assumed that they affect carotene absorption through the inhibition of lipase activity.

On the basis of our results the use of formic acid in canned products containing carotene appears worrying.

SUMMARY

1. The influence of formic acid, sorbic acid and Na benzoate on the utilization of carotene was studied in white male rats.

2. The investigated preservatives had no effect on weight gain of the experimental animals and the histological examination of liver and intestines showed no pathological changes.

3. In the formic and sorbic acid groups the stored vitamin A was smaller than in the controls after 2, 4, 6 weeks administration of carotene. The difference was most significant in the 2 weeks experiment. Na benzoate had no effect on the vitamin A content of the liver.

4. Carotene-balance tests showed that formic and sorbic acids increase

the carotene excretion and the deterioration of carotene utilization is probably due to that fact.

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PHYSIOLOGICAL AND MORPHOLOGICAL CHANGES IN RATS FOLLOWING PERORAL ADMINISTRATION OF BENZOIC ACID.

H. Kreis, K. Frese & G. Wilmes

(Fd. Cosmet. Toxicol. 5: 505-511, 1967.)

INTRODUCTION

Research concerning the toxicity of benzoic acid has repeatedly established that exceeding a certain daily dosis in test animals will cause the occurrence of fairly definite clinical symptoms. These essentially consist of central-nervous disorders with increased irritability and tonic-clonic cramps comparable to human epilepsy (Rost etal, 1913).

However the literature contains only scant information concerning anatomical or histological tests determining benzoic acid damages. In particular, central nervous research has not yet been performed.

The described research in the toxicity of benzoic acid as carried out on rats attempted to determine whether besides damages that can be shown by means of "physiological criteria" such as weight development, feed effects, behavior etc., there are also histological changes, and if so, if as a function of time. Past research attempted to clarify:

(1) Following oral intake of toxically acting doses of benzoic acid, will there be morphological changes too and from what time on may they be shown?

(2) Do short-term intakes of benzoic acid cause damages extending beyond the duration of administration?

(3) Do even small amounts of benzoic acid doses cause damages?

EXPERIMENT

Growing male rats were used as test animals (Royal Wister; breeder: Meyer Arend, Bad Salzuflen) with an initial weight of about 60 gm on the average. These animals were kept in individual cages with wire mesh floor.

Test-feed consisted of half-synthetic meals as used by HOETZEL (196 60.0 gm of saccharose (half of it ground): 5.8 gm of rice starch (DAB 6 20.0 gm of casein (vitamin free): 7.0 gm of purified butter fat, includ vitamines A, D and E; 4.0 gm of mineral substance mixture USP XIII, liquid; o.2 gm of methionine; 1.0 gm of rice starch + 0.2 gm of choline chloride; 2.0 gm of rice starch with vitamins B.

A special feed mixer (LOEDIGE, Paderborn, Model F 20 E) was used for mixing the feed components and, to the extent required, the benzoic acid addition. Drinking water and food were administered ad libitum.

The wire-mesh feeding method described by HUELLER & HOETZEL (1955) prevented the usual strewing of feed with rats to a large extent. Thus the actual feed intake could be fairly accurately determined.

During the entire test duration there occurred two weekly weight measurements. The testing plan shown in table 1 shows the testing program. In order to correspond to individual problem-statements, the program was divided into three series (A,B,C) and a fourth one was used as control (K). For 3% of benzoic acid in the test feed,(series A), the animals will die, as is known, after a few days. In order to determine after which time organic damages first occur for 3% benzoic acid in the feed, certain animal groups were examined at given time intervals. The animals in the B-group (also subjected to 3% benzoic acid) were supposed to receive benzoic acid till all animals reliably had suffered damages. This time-limit was taken to be 5 days, since at that time about 80% of the animals had died. The surviving animals of group B1 were killed on the 6th day. The animals surviving the 5th day in group B11 were put on a normal diet (no benzoic acid) in order to determine damages extending beyond duration of administration.

Table 1: Testing program for benzoic acid administration
GRUPPE: group; ANZAHL: number of animals; IN FUTTER: in feed; GABE: administration; TAGE: days; VERSUCHSDAUER: test duration; BENZOESÄURE: benzoic acid.

Gruppe	Tierzahl	Benzoesäure % im Futter	Benzoesäure Gabe (Tage)	Versuchsdauer (Tage)
A I	5	3	1	1
II	5	3	2	2
III	5	3	3	3
IV	8	3	5	5
B I	10	3	5	5
II	15	3	5	24 bzw. 35
C I	5	1,1	7	7
II	8	1,1	14	14
III	10	1,1	35	35
K I	5			3
II	7			35

The animals in series C received a tolerable dosis of 1.1% benzoic acid. In order to detect any damages, these animals were killed several weeks apart. Both control groups (series K) were used in addition for the comparison of physiological criteria.

The surviving animals of the individual test groups were killed by decapitation and were immediately dissected, or as soon as possible for the rats. Histological examination of heart, liver and kidneys occurred after fixing in 10% formaline for frozen sections which were dyed red

with hematoxiline and scarlet red for fat-representation. After careful preparation, the brain would be removed from the skull cavity and be fixed in 10% formaline, and after dissection into frontal slices would be embedded in paraffine. Evaluation essentially was performed from sections dyed with hematoxiline-eosine and cresyl violet.

RESULTS AND DISCUSSION

For 3% benzoic acid in the feed, most animals after 4-5 days would show abnormal behavior (Groups A IV, BI and BII). Initially one observed a stiff, slightly ataxic walk soon followed by more or less pronounced shaking of the entire body. Simultaneously one could observe signs of motor unrest which subsequently were reinforced. The animals ran restlessly in the cage or executed aimless motions, some even carrying out manège motions. Frequently there was increased aggressiveness in that the animals suddenly bit the cage grille or objects tendered to them. Most animals were remarkable fearful, to the extent they would react by fear-motions or by reactions to touch or to normal lab room noises. The tonic or tonic-clonic cramps setting in thereafter were characteristic, to the point that sometimes there was strong extension of the rear limb muscles. Meanwhile the cramps did not last long, but they occurred frequently in many animals. Thereafter the animals fell into a somnolent state that most of the time was shortly followed by death. Most of the time only hours would elapse between the first signs of benzoic acid intoxication and death, and never more than one day (full) after five days about 50% of the animals had died (Groups A IV, BI and B II; 3,4 and 7 animals, resp.) with the symptoms as listed. Among the surviving rats, abnormal behavior -- notably the cramp-like symptoms - were not as pronounced or frequently noted.

The research in the toxicity of high dosages of benzoic acid essentially confirms the results of other authors. HITCHCHUSCH & LEE (1960) found that rats perish if fed 3% benzoic acid within 14 days. HOFTEE (1962) stated that the LD_{50} for benzoic acid is 1.5-2.0 gm/kg of body weight for rats. Kiesewetter et al (1963) observed diseases in dogs similar to human [illegible] repeated administrations of 1 gm of benzoic acid per kg of body weight. Besides unrest, ataxia and increased aggressiveness, fits of clonic cramps occurring hours and days apart were characteristic. Thus one animal showed 22 fits in 14 days. Exhibiting the characteristic cramps following administration of high benzoic acid doses followed within a few hours by death: Kiesewetter et al (1963). Griffith (1962) experimented with 3.5% sodium benzoate in feed and found that unrest, ataxia and cramps preceded the rats' death. If several animals were kept in one cage, increased aggressiveness

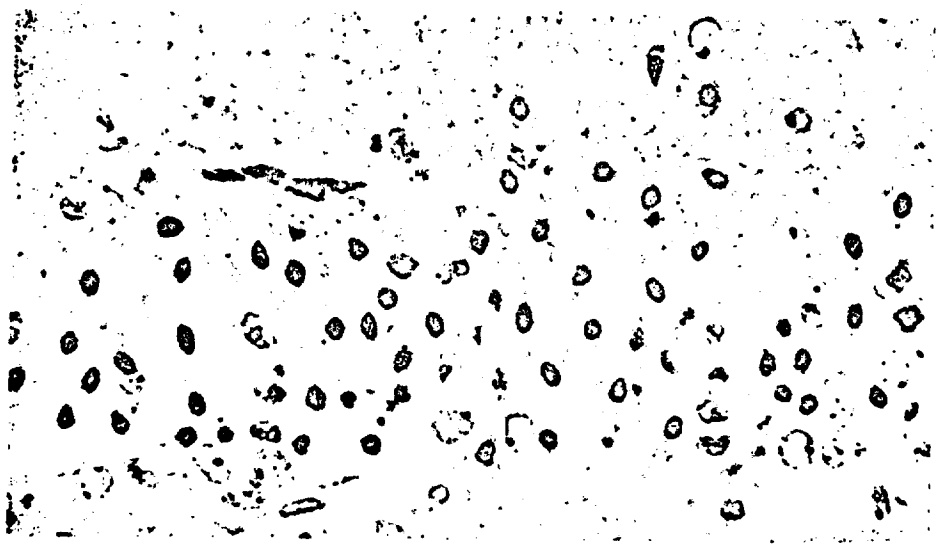

Fig. 1: Ischemic ganglic cell necrosis in stratum granulosum of the fascia dentata of hippocampus formation after 5-day feeding of 3% benzoic acid. Clear pycnosis of the cell nuclei and shrinking of the perikaria. Rat II/2. HE dyeing. Leitz Orthomat, lens 40:1

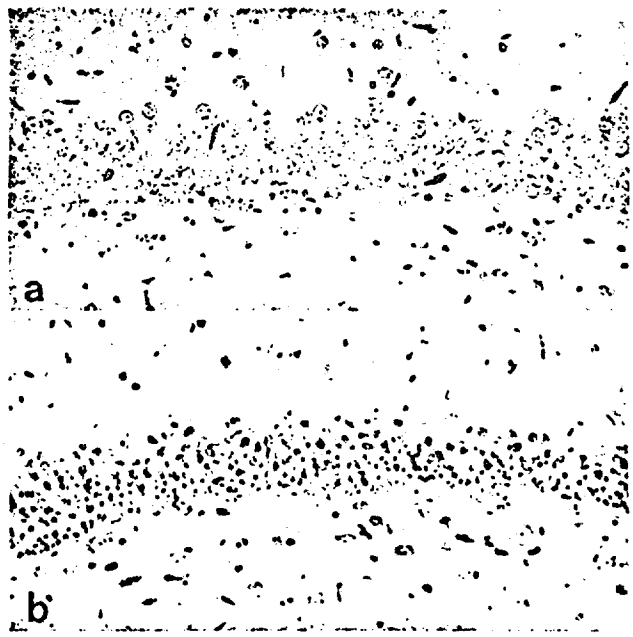

Fig. 2 : section from stratum granulosum of the fascia dentata of the hippocampus formation: a) from a healthy rat : b) from a rat after 5-day feeding with 3% benzoic acid. Ischemic necrosis of most ganglic cells without significant changes. Rat [illegible] II/2[illegible]. HE dyeing, Leitz Orthomat, lens 10:1.

One may therefore state in conclusion that peroral administrations of strongly toxic benzoic acid doses will lead to central nervous disorders expressed by movement disorders, increased excitability and cramps. While no surprising changes in organs could be observed for the sections of the animals fed with 1.1% benzoic acid, those rats fed with 3% benzoic acid in their feed from the 3rd day of administration in individual cases suffered from bleeding in the intestine and also frequent if light tympany of the small and large intestines, more

[illegible] spleen and the kidneys of these animals could be ascertained. Histological examination in all groups of heart and kidneys showed no suspicious changes due to benzoic acid. The livers did show frequent peripheral small-drop fat infiltration of the patches, also slight histiocytic-cellular periportal infiltrates and occasionally submiliary granulomas mostly associated with the periportal fields. These however were irregularly distributed in all groups including the control groups.

On the other hand noticeable findings were made in the brains. The frequency of these changes is listed in table 2.

Table 2: Frequency of brain changes following benzoic acid feeding.

Group	frequency	group	frequency
A I	0/8	CI	0/5
II	0/8	II	0/8
III	0/8	III	0/10
IV	3/8		
B I	10/10	CI	0/5
II	13/15	II	0/7

Whereas no damage to the central nervous structures could be ascertained at all in the animals of groups CI-CIII nor for those in the control groups, damages could be shown already after three days of benzoic acid feeding (3) in 2 cases and after 5 days of feeding in all cases. Also, among the surviving animals of group B II which thereafter received no benzoic acid for 10 or 30 days, brain changes could be observed every case.

The histology was characterised by the occurrence of characteristic ganglia cell changes in the form of so-called ischemia ganglia cell necrosis, which will be briefly outlined. There was considerable shrinking of the periphery of the ganglia cells which showed more or less pronounced eosinophilia in haustoxiline-eosine dyed preparations though only weakly tinting in the cresyl violet dye. Chromatophilous corpuscles could not be found. The cell nuclei too were strongly shrunk and in most cases looked as if karyohectic decomposition into more or less fine chromatine particles had occurred, nuclear shape still possibly being maintained in the case of finer particles. Simple nucleus pycnosis generally was rarer but in individual cases might be preponderant (fig. 1).

The pycnotic nuclei were round-oval, also in the shape of pyramid-cells and occasionally triangular. Among those animals that had died within 3-10 days of initial feeding of benzoic acid feed, or which had been killed, the nuclei or their decomposition products were dyed in

a more or less distinct tint with cresyl violet coloring. On the other hand, the animals killed after 24 or 35 test days, from group B II, had nuclei that could hardly any longer be colored by means of the basic dyes and looked blue red. One could not determine with certainty whether there had been reactive changes in the form of glia proliferation among the animals of groups A III-A IV and B I and among the rats which had been examined early in group B II. In no case could one observe vascular changes and the occurrence of grain cells. Also there were no apparent damages in the marrow substance. The distribution pattern of the described change in ganglia cells in all cases was of striking similarity. The fascia dentata of the hippocampus formation were constantly affected; in most cases a nearly complete loss of ganglia cell in the stratum granulosum could be observed (fig. 2).

Changes of nearly the same frequency if of lesser extent were found in the cortex of the lobus piriformis (area prepiriformis, front and temporal field in the nomenclature of [illegible] & [illegible] (1963)). More rarely, in 2 cases of groups B I and B II, there were individual ganglia cell necroses also at the so-called end leaf of the Ammons horn. In three cases there were slight cell damages or destruction also in the area of the dorsal pyramid cell band of the hippocampus, a domain in which according to [illegible] (1[illegible]) the [illegible] sector (field h 2) is comparable to that of man's. In the remainder of the large cortex, in the basal ganglia, in the mid-brain and in the cerebellum including the medulla oblongata, there was absence of noticeable changes. It should be noted however that on other grounds it was impossible to histologically treat the brains in a series of step sections and that complete information therefore cannot be made as regards the distribution pattern.

As already emphasized, there is only little information in the literature concerning the pathomorphological findings when benzoic acid is administered. [illegible] (1[illegible]) failed to show a carcinogenous effect for benzoic acid. According to [illegible] & [illegible] (1960), there were no indications of harmful effects from 16 weeks of feeding rats with 0.5% or 1% benzoic acid addition from a histological examination of their organs. This information may be corroborated by our own tests with 1.1% benzoic acid in the feed (groups GI -GIII). Our own work further shows that for peroral administration of 3% benzoic acid addition unspecific histological changes may not be observed in the heart, liver, and kidneys of rats.

However there was clear damage to these animals' brain parenchyma. The damage essentially consisted in changes in ganglia cells which, because of their morphology (cell shrinkage with nuclear pycnosis and [illegible] nuclear destruction, plasma eosinophilia and loss of

dyeing affinity for cresyl violet) may be interpreted as ischemic ganglia cell necrosis. Noticeable changes in the marrow substance and softening and indications of cerebral edema in no case could be demonstrated. The reaction changes, particularly so for the surviving animals of group B II, among whom examination was easier, consisted of a little pronounced glia proliferation: vascular reactions could not be demonstrated. Hence the damages that were observed may be ordered in the sense of the parenchyma necrosis of SCHOLZ (1957). These are characterized by the occurrence of ganglia cell necrosis without there being strong damages to the other structures in particular the glia. The elective parenchyma necrosis represents tissue damage occurring upon acute and temporary lack of oxygen most of all, and also when there are other disorders in tissue respiration. Questions arising in this respect in the field of pathogenesis for the individual findings cannot be answered more fully within the scope of this work. After LANG's compilation (1960) concerning the inhibition of ferments and the biochemical processes due to benzoic acid, there is no ground that benzoic acid may exert toxic effects on the brain via a specific inhibition of ferments in the sense of a lack of working substance hypoxidose. SCHOLZ particularly (1957) and more recently PEIFFER (1963) have interpreted the significance of cramps in the generation of parenchyma necrosis. The kind and the distribution pattern of the brain damages observed in this research, showing a preponderance of hippocampus changes and laminar ganglia cell necroses in the cerebrum cortex, show certain similarities with cramp damages in the human brain, where again the changes in the Ammons horn are preponderant. The fact that cramps were the characteristic clinical symptom among the rats of our research makes us assume they were at least contributing to the cause in ganglia cell changes. Obviously the described changes in the brain tissue do not necessarily lead to the death of the test animals. Even though all animals had suffered brain damage after being fed for 5 days with benzoic acid feed (B I), the majority of the animals will survive if thereafter they are provided food without benzoic acid, B I Nevertheless, as had been expected, the brain damage could still be demonstrated 19 or 30 days after they had been placed on diet free of benzoic acid. The five-day addition of benzoic acid to the feed also seems to have adversely affected the weight evolution. The difference with respect to the control group K II however could not be ascertained with sufficient reliability in view of the small numbers involved, but the weight increase in this group was even smaller than in group C III, which statistically reliably does differ from the control group.

The groups of the C series received 1.1 % of benzoic acid in their feed, a concentration exceeding the toxic limit. Group C III showed a significantly poorer increase in weight and in response to feed than did the control group [illegible] (170.[illegible] ± 17.1 gm as compared to 196.7 ± 20.7 gm , or 0.335 ± 0.032 as compared to [illegible] ± 0.[illegible]). HOTZEL (1962) observed a growth decrease in rats following administration of 1 gm of benzoic acid per kg of body weight; [illegible] (1960) for 1 % of benzoic acid in feed observed however no adverse effects. However a dosage of 1 % may be considered to be close to the upper limit of tolerance, as shown by our own results and the work of other authors ([illegible], 1960; [illegible], 1960; [illegible], 1960). This is further confirmed from the observation that a dose of about 1 % of benzoic acid in the feed does not lead to changes in behavior or to organic damages observable by histology.

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ON THE FATE OF C^{14} BENZOIC ACID AND C^{14} P-CHLOROBENZOIC ACID IN THE ORGANISM

H. Lang &K.Lang; 3 figures.

In connection with the use of benzoic acid or of substituted benzoic acids as chemical preservatives, there is the question of a possible storage of those substances in the organism and of their behavior in the metabolism.

It has long been known that the incorporated benzoic acid is bound to glycocoll and hippuric acid and will be eliminated rapidly and and completely in this form in the urine. BERNHARD ETAL. (1) recently showed that when using C^{14} benzoic acid, that such will be early completely eliminated through the urine within 24 hours of peroral or subcutaneous administration as benzoic acid. They further made it seem plausible that benzoic acid is not modified in the body. The carbon dioxide expired was radioactive only to a minor extent, and the urine eliminated benzoic acid had the same deuterium content as the substance administered except for the isotope dilution from metabolic self-production. These tests however did not exclude the feasibility of benzoic acid enrichment in certain organs, all the more so that the partition coefficient of the substance between water and organic solvents makes a storage in lipoid-rich organs quite feasible. We therefore attempted to settle the question of a possible storage of C^{14} benzoic acid and in the process we extended our investigations to p-chlorobenzoic acid.

METHODOLOGY

1. Benzoic acid preparations. Benzoic acid ($C^{14}OOH$) with a specific activity of 10.8 mC/g and p-chloro-benzoic acid ($C^{14}OOH$) with a specific activity of 13.7 mC/gm were used.

The weighed parts were dissolved in water after careful addition of 0.1 NaOH and adjusted for a pH of 7.0-7.5. After filtration through a frit G4, the solutions were stored in a refrigerator. The 50 mg dose was contained in 0.65-1.0 solution. Aliquot samples were taken from the solutions and diluted in measuring flasks; several measurement preparations for standardizing the solutions were dried each with 1 ml

of saturated $Ba(OH)_2$ solution and then were measured.

2. Radioactive measurements. The measurements were performed with a terminal window counter tube from the firm of FRIESEKE & HOEPFNER with a glow foil of 1.6 mg/cm2. Screening, identical positioning and similar spacing of preparations were precisely ensured. The counter tube output was checked by means of a uranium and C^{14} standard. Network fluctuations were eliminated by repeated measurements.

3. ANIMAL TESTS. Albino rats of both sexes weighing from 140-230 gm were used. The animals received doses of 50 mg in 0.65-1.0 ml, which had been pre-heated to body temperature and which were injected into the abdominal cavity by means of a tuberculin syringe. Until killing, the animals were kept in a well aerated location in metabolism cages; feces were caught by means of a fine-meshed wire sieve, and urine was collected in a Erlenmayer flask containing 5 ml of 1 n NaOH. Feces and urine were removed daily.

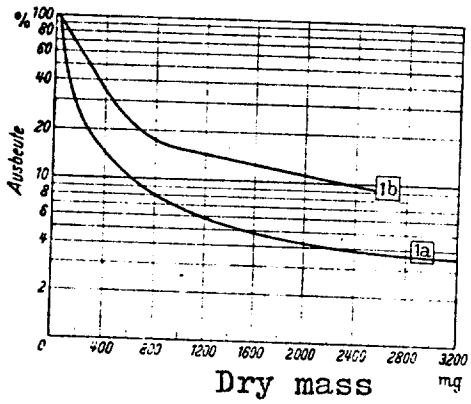

Fig. 1. -1.a- Self-absorption curve for C^{14} in dried rat tissue homogenates . 100% yield for nominal with 37 mg of dry mass. -1b- Self-absorption curve for C^{14} in the dry residues of ether extracts in rat tissues. 100% yield for nominal with dry mass less than 20 mg.

4. PROOF OF RADIOACTIVITY IN THE WHOLE ORGANS. The rats were killed by neck blow after various times (1-23 days) and were bled by cutting open the carotis. The corpses were immediately dissected and after flushing with water were processed further. Preliminary tests having shown that the radioactivity of the individual tissues was too small to them following incineration and precipitation of CO_2 as $BaCO_3$, the radioactivity of the homogenates of the tissues was directly measured despite theoretical reservations. A calibration curve was used for correcting self-absorption, by mixing various amounts of homogenates from various organs with 1 ml each time of saturated $Ba(OH)_2$ solutions and 0.054 uC C^{14} benzoic acid in properly weighed flat

glass dishes of 40 mm diameter and then drying them at 70°C. Prior to complete drying the layer was smoothed by adding each time 1 ml of acetone by means of a stockcock , whereupon drying was completed. From the measurement of 35 different samples with dry masses of 35-4300 mg and from different organs, a satisfactorily accurate curve for the self-absorption of C^{14} was obtained for the rat tissue homogenates; see fig. 1a. All homogenate values satisfactorily lay on the curve and the mean deviation of the the measured values from the curve amounted to ± 5.1%. The activities of the samples without tissue and with only 1 ml of saturated $Ba(OH)_2$ solution and 0.054 uC C^{14} benzoic acid (mean dry mass: 37 mg) were used as the value for 100% yield, extrapolation of the curve to 0 mg being too inaccurate in view of the steepness of the curve. The rat tissues were homogenized totally in the whole-glass homogenizer or they were partly homogenized with weighed, aliquot parts with addition of 1 ml of saturated $Ba(OH)_2$. Tougher tissues such as musculature etc. were previously minced by means of scissors in a porcelain dish. Bones were reduced after addition of $Ba(OH)_2$ solution by means of pestal in a grinding dish and ground to as fine a mash as possible. Feces were well homogenized and filled to 100 ml with water and the well stirred suspension was analyzed in lots of 1 ml each. Urine was filled to 500 ml and was tested several times in amounts of 0.5 ml. Skin activity could not be measured in this fashion. All homogenates were treated precisely as described for setting up the calibration curve and were measured in that manner. Tables 1 and 2 show the results.

5. PROOF OF RADIOACTIVITY IN THE ETHER EXTRACTS OF RAT ORGANS. The test animals were killed and dissected as described in 4; the tissues were transferred in toto or in aliquot parts to glass goblets and hydrolyzed with 10% NaOH for one hour at 100°C, the vaporizing water being replaced. The hydrolysates practically no longer contained solid particles and after cooling were placed into KUTSHCER-STEUDEL extractors and were acidified with 20% HCl and extracted for 20 hours with 50 ml of pure ether. The ether phase was dried with annealed Na_2SO_4 for 24 hours and then it was filtered, the Na_2SO_4 residues being well rinsed with 50 ml of dry ether. Then the united

solutions were evaporated in vacuum on the sand bath at about 40°C, until there was a residue of 2-3 ml; the latter was transferred to dishes of known weight and the flasks were repeatedly flushed with 1 ml each of dry ether. After ether evaporation, the layer was flattened by adding 1 ml of dry acetone and dried at 70°C, then measured after weighing. Results are listed in table 3.

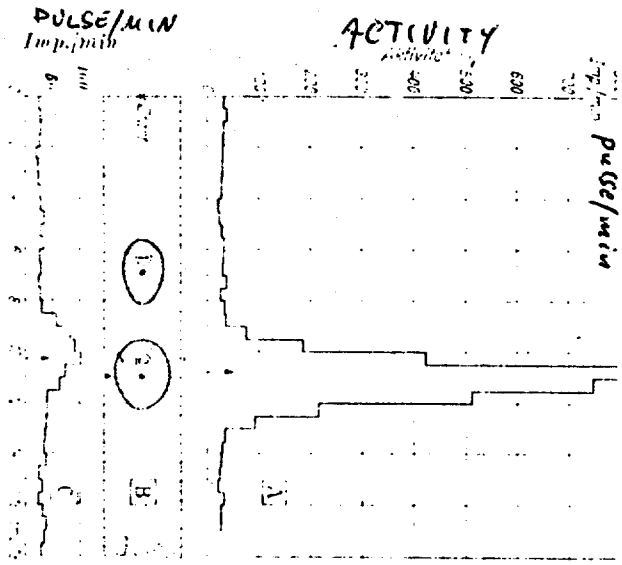

Fig. 2. Paper chromatograph of ether extract from rat tissues (see text for method). A.- Chromatogram radioactivity from 7 gamma C^{14} benzoic acid. B.- reproduction of chromatogram of (1): 50 gamma hippuric acid and (2) 50 gamma benzoic acid. C.- chromatogram radioactivity of purified rat tissue ether extracts.

Here use was also made of a self-absorption calibration curve by filling the extractors with increasing amounts of NaOH hydrolysates from various organs each with 0.054 uC of C^{14} benzoic acid and after acidification, as described above, drying, filtering and evaporating the ether phases. This self-absorption curve for C^{14} in the residues of ether extracts from rat organs is not identical with the self-absorption curve of the homogenates (fig. 1b). Here too all measured values -- 13-- lay satisfactorily close to the curve. The 100% yield was taken to be the activity of the residues of 0.054 uC C^{14}benzoic acid and of 10 mg of inactive benzoic acid lacking a tissue hydrolysate (mean dry mass: 8 mg).

6. PAPER CHROMATOGRAPHIC EXAMINATION OF ETHER EXTRACT RESIDUES.

The residues from ether extraction in the measurement dishes were again dissolved in ether and collected. The organic values from four rats were combined. These ether solutions show yellow precipitates upon standing and they were shaken out three times with saturated

$KHCO_3$ solutions, the two phases becoming wholly clear, so that no insoluble residues remained. The combined aqueous phases were rendered alkalineby means of three drops of 10% NaOH and they were shaken out backwards three times with ether in order to separate as much fat as possible. The aqueous phases were acidified with 20% HCl and shaken out twice with ether; the ether solutions were combined and were once more subjected to the just described procedure, as were the initial solutions. The resulting, now almost non-adipose ether solution was dried over night with annealed Na_2SO_4 and dried under vacuum almost to the dry state. The residue, a yellow-brown oil, was absorbed in some ether and deposited almost quantitatively on the paper chromatogram. A one-day amount of rat feces also was hydrolyzed with NaOH, then extracted by means of ether and processed according to the manner described above and the residues of the $KHCO_3$ extract were chromatographed. Chromatography takes place with Whatman Nr 1 solvent n-butanol, saturated with 1.5 n NH_4OH, decreasing direction of motion, time of operation: 20-26 hours, temperature: ca 25°C. 50 gamma each of

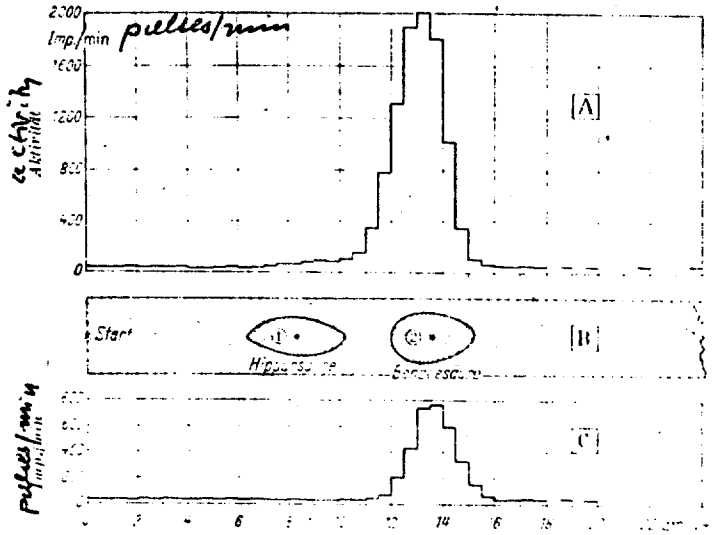

Fig. 3. Paper chromatography of rat feces ether extract. A.- radioactivity in the chromatogram of the purified rat feces ether extract. B.- reproduction of chromatogram from a) 50 gamma of hippuric acid and b) 50 gamma benzoic acid. C.- radioactivity in the chromatogram of 7 gamma of C^{14} benzoic acid.

benzoic and hippuric acids and 7 gamma of C^{14} benzoic acid were chromatographed on the same sheet together with the empirical substances as comparison values. A strip 4 cm wide was cut out of the chromatograms to measure radioactivity and was tautly glued to an equally wide glass plate that was screwed into a metal rail which could be slid underneath the counting tube. The latter was screened by a 1 mm lead foil with a cut-out 5 mm wide and 25 mm long. A scale engraved into the metal rail allowed measuring the strips continuously in 0.5 cm spacings. Results are shown in fig. 2 and 3.

In order to check whether the $KHCO_2$ extraction took place quantitatively and whether any substance had been overlooked, the ether solutions remaining after $KHCO_2$ extraction were shaken out with water and each was filled to 20 ml and aliquot parts were examined for activity. It was found that there was 4% residual activity in the ether solutions from the organic extracts and 1% from the feces extracts.

RESULTS AND DISCUSSION OF FINDINGS

Table 1 lists our findings on the distribution of C^{14} benzoic acid following ip. injection in rat organs. In agreement with BERNHARD ETAL (1), our results showed that the benzoic acid administered will practically be urine eliminated within 1-2 days. We found only slight radioactivity in the feces. The radioactivity of the organs tested was extremely low. It was no more, order of magnitude-wise, than hundredths of a percent of the applied dose. Despite the high dosage, namely about 500 uC, in the animals weighing about 200 gm, radioactivity was found to be at the detectable threshold in the brain (10^{-4}% of dose). Radioactivity steadily decreased in the course of 8-23 days in the organs and then became practically undetectable. Somewhat higher though still vanishingly small activities could be observed in the fat deposits. Here too radioactivity decreased rapidly with time.

In order to determine whether the activity measured in the organs was due to the benzoic acid content or whether conversion products of same could be proven, we measured the activities in a second test series not in the organic homogenates but rather in the ether extracts of same. It was found that practically all of the homogenates radio-

activity had been transferred to the ether extract (see table 2). We then purified the ether extracts and subjected them to paper chromatography. This yielded only a few spots and because of its R_f value (control by means of concurrently running benzoic acid) was identified as benzoic acid (fig. 2 and 3). Thus the entire organ radioactivity and that of the fat deposit's is determined only by the benzoic acid content. Conversion of benzoic acid into other substances does not take place in cell metabolism. Any hippuric acid that is present however would have been saponified to benzoic acid in organic recovery. Therefore it is possible that benzoic acid is present in cells in the form of hippuric acid, in toto or in part.

Tests with C^{14} p-chlorobenzoic acid led to the same findings (table 3). P-chlorobenzoic acid too will be practically eliminated completely in the urine within one day. We found only 2% of the dose administered in the feces. Organic content too was of the same order of magnitude as after benzoic acid administration. Again radioactivity, despite the larger lipoid-solubility of the chlorinated acid, was nearly vanishingly small in the brain; it was largest in the fat deposit. Our findings are in agreement with results reported by VON HECHT and that were obtained from chemical analysis. HECHT too could observe no p-chlorobenzoic acid storage in the organism.

Organ	nach 1 Tag (AFTER 1 DAY)	nach 2 Tagen (2 DAYS)		nach 4 Tagen (4 DAYS)	nach 8 Tagen (8 DAYS)	nach 23 Tagen (23 DAYS)
Urin . . .	93	102	107			
	98	104				
Kot (FECES)	0,8	3,1	1,7	1,0	—	—
Blut (BLOOD)	0,009	0,004	0,007	0,002	≤ 0,002	≤ 0,001
Leber (LIVER)	0,02	0,009	0,008	0,004	0,004	≤ 0,0006
Nieren (KIDN.)	0,003	0,002	0,002	0,0008	≤ 0,0005	≤ 0,0001
Darm (INTEST.)	—	—	0,01	—	0,004	≤ 0,0003
Milz (SPLEEN)	—	≤ 0,0001	—		—	—
Lunge (LUNGS)	0,001	—	0,001	—	0,0001	—
Fett um Nieren (KIDNEY FAT)	0,02	0,03	0,02	0,02	0,01	0,002
Muskulature (MUSCULATURE)	0,05	0,04	0,03	0,08	0,03	0,007
Skelet (SKELETON)	—	—	—	0,008	≤ 0,003	—
Gehirn (BRAIN)	0,0003	0,0005	0,0002	0,0001	0,0001	≤ 0,0001
Haut (mit Fett)* (SKIN WITH FAT)	0,3	0,1	0,2		0,09	0,02

TABLE 1. Distribution of benzoic acid in rats following ip. injectio
Values in % of applied doses. Error spread: urin = ± 5%; feces = ± 20%
other values = ± 50%. Values recovered in homogenates.
* Skin values for ether extract, since no homogenate was feasible.

The results lead one to assume that oxybenzoic acid and p-chlorobenzoic acid are not absorbed in detectable amounts by organic cells, but rather remain largely extra-cellular. On the basis of the restrictic of the substances to extracellular domains, there will be rapid elimination into the urine. Pairing of glycocoll with hippuric acid occurs in the kidneys (2). Organic cell damage from benzoic acid or p-chlorobenzoic acid or appreciable influence of those substance as regards metabolism for the selected doses of 200-250 mg/kg therefore seems extremely unlikely, a detectable concentration not havi been achieved.

Organ	after nach 1 Tag 1day	nach 2 Tagen 2 days	
Urine	92	102	89
Kot feces	0,9	1,4	0,6
Blut blood	0,01	0,004	0,006
Leber liver	0,02	0,005	0,01
Nieren kidneys	0,009	0,009	0,002
Darm Intestine			0,03
Lunge lungs			0,001
Fett um Nieren Fat round kidn.	0,01	0,007	0,02
Skeleton	0,03		
Gehirn BRAIN	0,0003		0,0002
Haut (mit Fettschicht) SKIN WITH FAT LAYER	0,3	0,1	0,2

Table 2. Distribution of benzoic acid in rats after ip. application. Values in % of applied dose. Error spread: urine = ± 5%; feces = ±20%; other values = ±50%. Values found in ether extract.

Organ	after 1day	nach 2days	nach 8days
Urine	83 H		
Kot feces	2,0 H		
Blut blood	0,01 H	0,02 H	0,004 H
Leber liver	0,08 H	0,03 H	0,0 H
Nieren kidneys	0,02 H	0,009 H	0,003 H
Darm Intestine	0,05 H	0,02 H	0,009 H
Lunge lungs	0,003 H	0,002 H	
Fett um Nieren FAT AB. KIDN	0,0[illegible] H	0,04 H	0,04 H
Muskulatur	0,2 H	0,08 H	0,1 H
Skeleton		0,04 H	0,02 H
Gehirn brain	0,0009 H	0,00[illegible] H	0,0002 H
Haut (mit Fettschicht) SKIN WITH FAT LAYER	0,1 E	0,2 E	0,1 E

Table 3. Distribution of p-chlorobenzoic acid in rats following ip. injection. Values in % of applied dose. H = found in homogenate; E= recovered in ether extract. Error spread: urine: ± 5%; feces = ± 20 other values = ± 50%.

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Kinetics of Salicyluric Acid Elimination in Man

GERHARD LEVY, LEWIS P. AMSEL, and HOWARD C. ELLIOTT

Abstract ☐ The kinetics of salicyluric acid (SU) elimination were determined in healthy adult men from urinary excretion and (in one instance) plasma concentration data following intravenous injection of SU or oral administration of salicylate and subsequent blocking of further SU formation by benzoate. Similar results were obtained by the two methods. The elimination of SU is considerably more rapid than is suggested by urinary excretion data obtained after oral administration of SU in aqueous solution. The kinetics of SU formation from salicylate can be characterized adequately by considering the formation process as the rate-limiting step in the excretion of SU after salicylate administration.

Key phrases ☐ Salicyluric acid—elimination kinetics ☐ Benzoic acid inhibition—salicyluric acid formation ☐ Excretion rates—salicyluric acid, as test drug, as metabolite ☐ Salicylate excretion—salicyluric acid formation rate effect

Knowledge of the pharmacokinetics of salicyluric acid (SU), the major metabolite of salicylic acid, is desirable for a more complete understanding of the kinetics of salicylate elimination in man. The relatively unusual dose dependency in the elimination kinetics of salicylate is due to the limited capacity of man to form SU (1-4). Urinary excretion data have been used to describe the kinetics of SU formation since there was evidence (4, 5) that the appearance of SU in the urine is rate limited by its formation. The purpose of this study was to determine the kinetics of SU elimination. Since SU is almost completely excreted intact in the urine, its renal excretion rate constant is practically equivalent to the overall elimination rate constant. Presented here are the results of experiments in which SU was administered orally and by intravenous injection. Reported also are data obtained by administering salicylate and blocking SU formation after some time by administration of a competitive inhibitor. The blocking method is of special interest in that it may be useful for assessing the elimination kinetics of certain other types of metabolites, such as glucuronides and sulfates, which are not readily available or cannot be administered as such for other reasons.

EXPERIMENTAL

The subjects were adult men in apparent good health. SU alone or with benzoic acid was administered orally in the morning on an empty stomach. In other experiments, salicylic acid (a direct precursor of SU in man) was given, and 3 hr. later the further formation of SU was blocked by administration of a large dose of benzoic acid. All drugs were given in solution as the sodium salt. SU was administered also by rapid intravenous injection of a solution in pH 7.2 phosphate buffer. Urine collections were made at frequent intervals after drug administration. In one instance, several blood samples were also obtained. SU in the urine and plasma was determined by methods described elsewhere (6-8).

RESULTS AND DISCUSSION

The excretion rate of SU after oral administration of this metabolite decreased exponentially with time and it was not or only slightly affected by concomitant administration of benzoic acid (Figs. 1 and 2; Table I). "Apparent"[1] elimination rate constants determined from the slopes of log excretion rate *versus* time plots ranged from 0.73 to 1.3 reciprocal hr., in good agreement with results reported from another laboratory (2). Ninety percent or more of an oral dose of SU was usually recovered unchanged in the urine and an additional ≃5% was excreted as an acid-labile conjugate. The excretion rate of SU also declined exponentially with time after benzoate administration in experiments where the subjects were given salicylate as a source of SU. However, the elimination rate constants thus obtained were considerably larger than the apparent constants derived from the experiments where SU was administered orally as such (Figs. 1 and 2; Table I). Benzoate served to block the formation of SU (9) rather than its excretion; it may be noted in the experiments with salicylate that there was no rebound or secondary rise in SU excretion after recovery from the effect of benzoate (inset in Figs. 1 and 2). An inhibition of excretory function would have caused accumulation of SU, resulting in higher excretion rates of this metabolite after elimination of the inhibitory agent than prior to its administration.

Data obtained by intravenous injection of SU yielded essentially the same elimination rate constant as was obtained from the experiment with salicylate and benzoate in this subject (Fig. 3). The re-

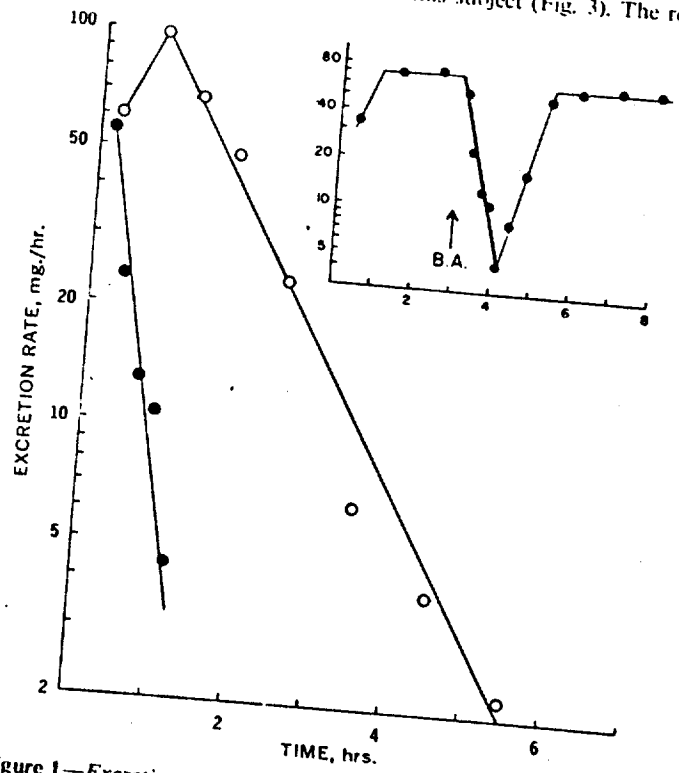

Figure 1—*Excretion rates of salicyluric acid as a function of time (a) after oral administration of 0.2 g. salicyluric acid (○) and (b) after oral administration of 3.2 g. benzoic acid preceded by 1 g. salicylic acid 3 hr. earlier (●). Inset: Time course of salicylurate excretion in experiment (b) starting from the time of salicylate administration. The heavy portion of the curve is identical to the curve shown on the main graph. B.A. = benzoic acid. Data from Subject A.*

[1] In the context of this discussion, the term "apparent" refers to the assumption, subsequently shown to be erroneous, that the slope of the log excretion rate *versus* time plot obtained after oral administration of SU can be used to calculate the elimination rate constant of this metabolite.

Table I—Elimination Kinetics of Salicyluric Acid (SU) When Administered Orally as Such and When Formed *in vivo* from Salicylic Acid (SA)

Subject or Reference	Exptl. Conditions	*Apparent* SU Elimination Rate Constant, hr.$^{-1}$ [a]	SU Elimination Rate Constant, hr.$^{-1}$ [b]
A [c]	0.2 g. SU	0.80	
A	0.2 g. SU and 5 g. BA [d]	0.73	
A	1 g. SA and 3.2 g. BA 3 hr. later		2.8
A	As above, with glycine [e]		2.8
A	1.5 g. SA and 5 g. BA 3 hr. later, with glycine [e]		2.6
B [f]	0.1 g. SU	1.0	
B	0.5 g. SU	1.3	
B	0.15 g. SU and 5 g. BA	0.78	
B	1 g. SA and 5 g. BA 3 hr. later		4.3
Reference 2 (2 subjects)	0.5 g. SU	≃0.83	

[a] When SU is given. [b] When SA is given. [c] 26 yr., 90 kg. [d] BA = benzoic acid. [e] 5 g. at −1 hr. and 2 g. every hr. thereafter. Glycine has no effect on SU formation in man and was administered in order to determine its effect on BA elimination (9). [f] 40 yr., 81 kg.

covery of intravenously administered SU was 96% of the dose and, contrary to what is observed following oral administration (10), there was no evidence of an SU conjugate. The plasma concentration data, though possibly not very precise due to an unusually high blank (about 0.18 mg./100 ml.), agree well with the urinary excretion data. The similarity of the elimination rate constant for SU obtained by intravenous injection of SU and by administration of salicylate followed by benzoate indicates that the considerably lower *apparent* elimination rate constants obtained in experiments where SU was administered orally are most likely artifacts resulting from slow absorption of SU from the gastrointestinal tract. Assuming that the slope of the line obtained by

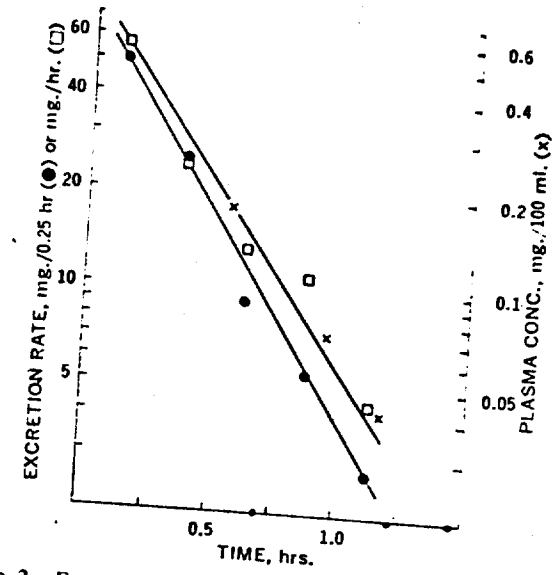

Figure 3—*Excretion rates (●) and plasma concentrations (×) of salicyluric acid as a function of time after rapid intravenous injection of 97.5 mg. salicyluric acid, Subject A. Shown also are the excretion rates of salicyluric acid after oral administration of 3.2 g. benzoic acid (at zero time) which was preceded 3 hr. earlier by 1 g. salicylic acid (□).*

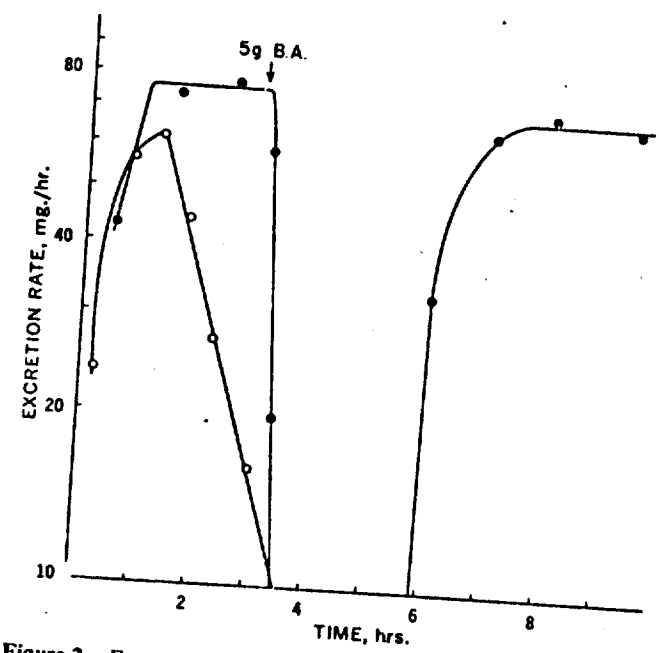

Figure 2—*Excretion rates of salicyluric acid as a function of time (a) after oral administration of 0.15 g. salicyluric acid followed 5 min. later by 5 g. benzoic acid (○) and (b) after oral administration of 1 g. salicylic acid followed 3 hr. later by 5 g. benzoic acid (●). A total of 11 mg. salicyluric acid were excreted during the 2 hr. when the data points from experiment (b) are below the lower limit of the graph. Data from Subject B.*

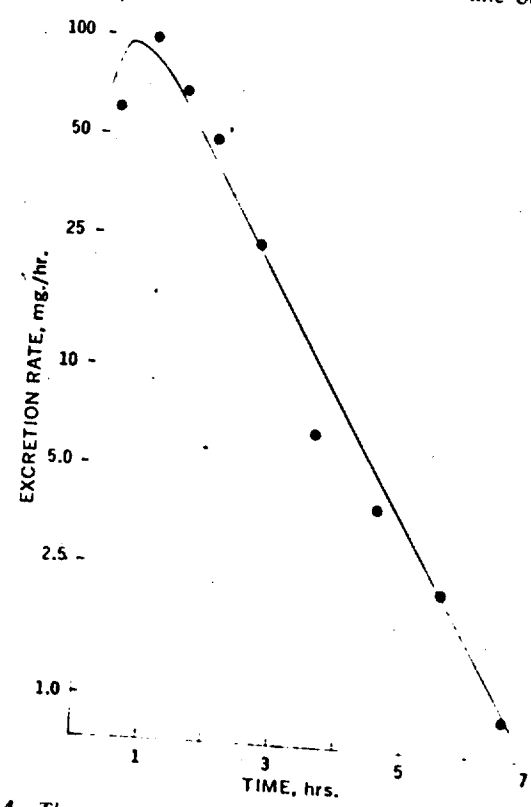

Figure 4—*Theoretical curve for a one-compartment model, assuming first-order absorption and elimination by excretion of orally administered salicylurate. The rate constants for absorption (0.8 hr.$^{-1}$) and elimination (2.8 hr.$^{-1}$) were calculated from the slopes of log excretion rate versus time data obtained after oral and intravenous administration of SU to Subject A. The experimental points are the same as in Fig. 1.*

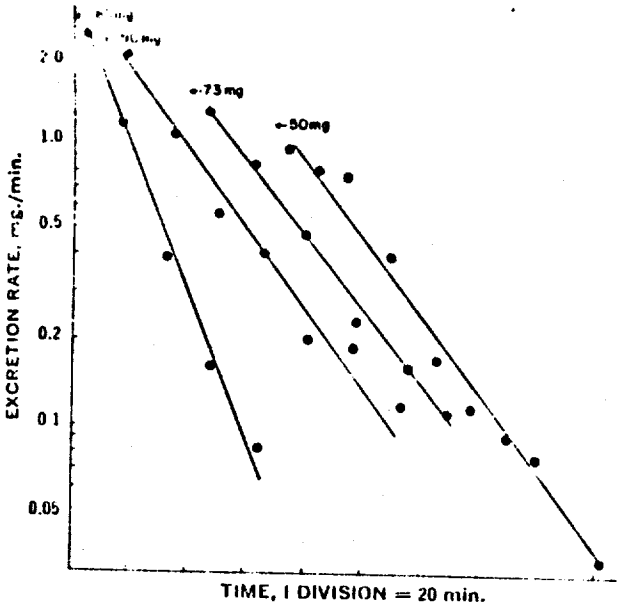

Figure 5 *Excretion rates of salicyluric acid by four healthy men as a function of time after rapid intravenous injection of salicyluric acid. The arrows indicate the time of injection and quantities next to arrows represent the administered dose.*

plotting log excretion rate of SU as a function of time after *oral* administration of this metabolite actually reflects the absorption rate constant (slope = $-k_{abs}/2.3$), and taking the slope of the excretion plot after *intravenous* administration to be a measure of the elimination rate constant, one obtains a theoretical curve which is in good agreement with the experimental data (Fig. 4).

The results of additional experiments with intravenously administered SU are shown in Fig. 5. These data were obtained as described by Elliott (11), but the present pharmacokinetic analysis was based on excretion rates and not, as in the cited paper, on plots of the difference between dose and the cumulative amount excreted at various times. The elimination rate constants for SU in the four subjects, calculated from the slopes of the log excretion rate *versus* time plots, are similar to that for Subject A (Table II). The largest value (3.5 hr.$^{-1}$ in Subject D) was similar to the 4.3 hr.$^{-1}$ value observed in Subject B in the blocking experiment. The observed rate constants for SU elimination exceed by a factor of 10 or more the rate constant for salicylate elimination determined on the basis of a one compartment model using data from small doses at which the kinetics are apparent first-order (12).

The very high elimination rate constant of SU (half-life approximately 0.3 hr.) found in this study is consistent with other evidence of rapid elimination of this metabolite, such as the existence of a linear relationship between salicylate concentrations in the serum or plasma and the amounts of salicylate in the body as determined from urinary excretion data (4, 5). The elimination (excretion) rate constant for SU is so much larger than the elimination rate constant of salicylate that theoretical considerations based on a one-compartment catenary chain model (13) indicate that SU excretion after salicylate administration is essentially formation rate limited. This conclusion is even more justified in the case of capacity-limited formation kinetics and makes it possible to use urinary excretion data for the determination of salicylurate formation rates and for establishing the relationship between these rates and the amount of salicylate in the body (5, 14). The use of the much smaller *apparent* elimination rate constant values (derived from experiments where SU was administered orally) for theoretical calculations leads to conclusions (13) which are inconsistent with evidence for saturation effects in salicylate elimination (15).

Table II Elimination Rate Constants for Salicyluric Acid after Intravenous Injection

Subject	Age, yr.	Wt., kg.	Dose, mg.	Elimination Rate Constant, hr.$^{-1}$
A	26	90	97.5	2.9
C	23	64	90.0	2.0
D	20	73	80.0	3.5
E	26	87	73.0	1.9
F	40	75	50.0	1.9

The synthesis blocking method used to determine the elimination kinetics of SU can also be utilized in principle for certain other types of metabolites. For example, it is feasible to block the formation of salicylic glucuronide with salicylamide (6) and it may be possible also to block the formation of phenolic sulfates with salicylamide (16). This may be particularly useful when a drug metabolite cannot be obtained or synthesized readily, or when it is unstable or poorly absorbed from the gastrointestinal tract and intravenous injection is not feasible. However, the possibility of an effect of the blocking agent on the distribution and/or elimination of the metabolite must be taken into consideration.

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ACKNOWLEDGMENTS AND ADDRESSES

Received July 29, 1968, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214* (G.L. and L.P.A.), and the *Departments of Chemistry and Medicine, University of Alabama, Birmingham, AL 35233* (H.C.E.).

Accepted for publication March 14, 1969.

Supported in part by grant 1 ROI DS00021-02 from the U. S. Public Health Service.

J. Pharm. Sci. 54(7):959-967, 1965.

Pharmacokinetics of Salicylate Elimination in Man

By GERHARD LEVY

Salicylate elimination kinetics was studied over a dose range of from 0.25 to 2.0 Gm. of aspirin. It was found that when the amount of salicylate in the body of normal adult test subjects exceeded approximately 360 mg. aspirin equivalent, conjugation of salicylic acid with glycine reached a maximum rate and thus proceeded by zero-order kinetics. The over-all elimination of salicylate was found to proceed by first-order kinetics at very small doses, and by parallel zero and first-order processes at higher doses. A kinetic model was developed, and values for appropriate rate constants were determined which make it possible to reconcile apparent half-lives for salicylate elimination ranging from about 3 hr. to over 20 hr. which have been reported in the literature. Unusual patterns in salicylate elimination kinetics observed by others, such as a change from zero to first-order kinetics upon concomitant administration of *p*-aminobenzoic acid, and zero-order elimination in a newborn infant, have been rationalized and interpreted by means of the kinetic model evolved from the present study. The pharmacokinetics of salicylate elimination were found to be unusual both qualitatively and quantitatively, and the results of the present study have potentially important therapeutic, toxicologic, and pharmacogenetic implications.

RELATIVELY little is known about the kinetics of salicylate elimination in man. This is not only unusual in view of the long and extensive use of salicylates, but also unfortunate since such information is necessary for establishing rational dosage regimens (1). For example, intensive salicylate therapy, as in the treatment of arthritis or rheumatic fever, frequently requires maintenance of plasma salicylate levels in a high but narrow range, and this can be accomplished only by means of a dosage schedule based on the elimination rate of the drug (2). Salicylate intoxication, particularly in children, is sometimes due to accumulation of the drug during therapy (rather than due to accidental ingestion) if the rate of salicylate administration exceeds the rate of its elimination from the body (3). Treatment of salicylate intoxication is directed mainly toward facilitating the elimination of the drug since all toxic manifestations are readily reversible with removal of salicylate from the body (4).

A review of the literature reveals that the apparent half-life of salicylate elimination increases with dose (Table I). The reported half lives range from 2.4 to 19 hr. in adults. The half-life of salicylate elimination in children intoxicated with this drug was found to range from 15 to 29 hr., with a mean value of 20 hr. (12). Similar results are evident from reported studies of urinary excretion of total salicylates (salicylate and its metabolites) as a function of dose (Table II), which show that the per cent of the dose excreted at a given time (prior to the completion of excretion) decreases with increasing dose. While an increase in half life with increasing dose is not unknown (13), it is a rarely observed phenomenon since the elimination of most drugs is describable by first-order kinetics with half-lives independent of dose.

Elimination of salicylate occurs mainly by the following parallel processes: renal excretion of unchanged salicylic acid, formation of salicyluric acid by conjugation with glycine, and formation

TABLE I. MEAN HALF LIFE OF SALICYLATE ELIMINATION IN ADULT HUMANS AS REPORTED IN THE LITERATURE

Drug	Dose Gm.	Dose mmoles	Route	Half Life hr.	Ref.
Sodium salicylate	0.25	1.6	i.v.	2.4	5
Aspirin	1.0	5.6	Oral	5.0	12
Aspirin	1.3	7.2	Oral	6.1	6
Salicylic acid	1.3	9.4	i.v.	6.1	[illegible]
Sodium salicylate	10.20	62.5-125	i.v.	19.0	8

Received February 26, 1965, from the Biopharmaceutics Laboratory, School of Pharmacy, State University of New York at Buffalo.
Accepted for publication April 20, 1965.
Presented to the Scientific Section, A.PH.A., Detroit meeting, March 1965.
This investigation was supported in part by grant RO1 AM 08753 01 PET from the U. S. Public Health Service, Bethesda, Md.
The author acknowledges the technical assistance of Mrs. J. A. Procknal and the cooperation of the volunteer subjects who participated in this study.

TABLE II. URINARY EXCRETION OF TOTAL SALICYLATE BY ADULT HUMANS AS REPORTED IN THE LITERATURE

Dose, Gm.[a]	% of Dose Recovered	Collection Period, hr.	Ref.
Sodium Salicylate			
1.0	78.6	24	(9)
2.1	54.5	24	
3.1	49.4	24	
4.2	50.8	24	
Sodium Salicylate			
0.5	82.7	24	(10)
1.0	78.6	24	
2.0	70.8	24	
Aspirin			
0.16	70.0	7.5	(11)
0.24	70.4	7.5	
0.32	71.5	7.5	
0.64	55.3	7.5	
0.97	52.4	7.5	

[a] Oral route.

of salicyl ester and ether glucuronides by conjugation with glucuronic acid (14). The purpose of the study to be reported here was to determine which of these processes was responsible for the observed decrease in the relative elimination rate of salicylate with increasing dose. In the course of this investigation it was found that the pharmacokinetics of salicylate elimination are most unusual and that their elucidation made it possible to rationalize and correlate numerous previously reported and often apparently contradictory findings.

METHODS AND EXPERIMENTAL PROCEDURES

The indicated amounts of aspirin or salicylic acid were administered orally in solution (dissolved in 200 ml. water by addition of equimolar amount of sodium bicarbonate) to healthy male subjects who had fasted overnight. No food or beverages were ingested by the subjects until at least 2 hr. after drug administration. Total urine collections were made at appropriate times. Blank urine samples were obtained immediately before drug administration and for an extended period of time after termination of drug excretion. In most experiments, urine collections were carried out for 24 hr. or more. The test subjects drank water at a frequency and in amounts sufficient to result in adequate urine output, usually 50–100 ml./hr.

Quantitative determinations of salicylic acid, salicyluric acid, salicyl glucuronides, and total salicylate in the urine were carried out by the method of Smith *et al.* (15) as modified by Levy (16). Urine pH was determined immediately after voiding (except in the case of urines collected at night which were stored in the refrigerator and pH determined the following morning). Analyses were carried out within 1 to 2 days after collection of urines. Up to that time, all urines were stored in the refrigerator.

RESULTS

The effect of dose on the metabolic fate of aspirin is shown in Table III. Almost all of the administered drug was recovered in the urine as salicylic acid or its metabolites. Urinary pH's, though variable, did not change with dose. The fraction eliminated as salicyluric acid decreased with increasing dose, while the fraction eliminated as unchanged salicylic acid and salicyl glucuronides, being due to parallel and competing processes, increased accordingly.

The time course of elimination of salicylate as a function of dose is shown for two representative subjects in Figs. 1 and 2, respectively. The first experimental point after zero time in each of these figures is affected by the absorption phase and is therefore not representative of excretion alone. All subsequent points represent postabsorption data since aspirin is rapidly absorbed when given in solution (17–19). Salicylate elimination did not follow first-order kinetics until the amount remaining in the body was decreased to about 400 mg. aspirin equivalent. Prior to that time (*i.e.*, in the case of the 1.0, 1.5, and 2.0-Gm. doses), elimination was not an exponential process (nor was it a zero-order process). The relative rate of salicylate elimination decreased with increasing dose, as is evident from the times necessary to eliminate half the administered dose. These times, indicated in the figures by vertical arrows, are somewhat affected by the absorption phase which tends to diminish the real differences. A better relative measure is obtained by subtracting about 0.5 hr. from each indicated time in order to account for the absorption phase. This will show that the time required to eliminate 50% of the administered dose increased from about 3 hr. with a 0.25-Gm. dose to about 9 hr. with a 2.0-Gm. dose. The first order elimination rate constant describing the *exponential phase* of drug elimination was essentially the same regardless of the amount of drug administered.

The time course of salicyluric acid excretion after administration of 1.5 and 2.0 Gm. aspirin, respectively, is shown in Figs. 3 and 4. These data were obtained from the same experiments as the data represented by the top curves in Figs. 1 and 2. Elimination of salicyluric acid proceeded at an essentially constant rate for over 12 hr., and this was a zero-order process during this time.

TABLE III. EFFECT OF DOSE ON METABOLIC FATE OF SALICYLATE IN HEALTHY ADULT HUMANS

Subject, Sex, Age	A, M, 36			B, M, 22			C, M, 23		D, M, 23	
Dose, mg.[a]	250	1000	2000	250	1000	1500	250	1000	250	1000
Amount excreted, mg.	232	928	1958	234	934	1490	236	935	215	92
Excreted as salicyluric acid, %	79.6	67.5	50.2	78.1	77.0	74.0	83.5	59.9	91.4	76.[illegible]
Excreted as salicylic acid, %	2.5	7.3	17.1	5.1	3.6	10.3	2.0	6.7	3.9	3.[illegible]
Excreted as salicyl glucuronides, %	1.9	25.2	23.7	16.8	19.4	15.7	14.5	33.4	4.7	19.4

[a] Aspirin taken orally. [b] Total salicylate as aspirin equivalent.

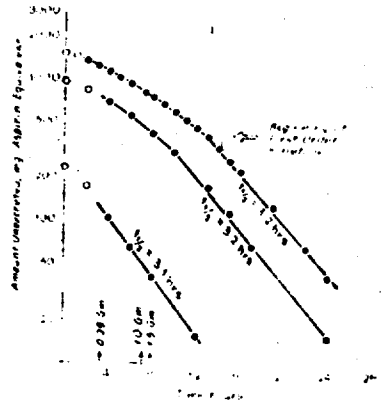


Fig. 1.—Elimination of salicylate by subject B as a function of dose. Doses taken were 0.25, 1.0, and 1.5 Gm. aspirin, respectively. Vertical arrows on the time axis indicate the time necessary to eliminate 50% of the dose.

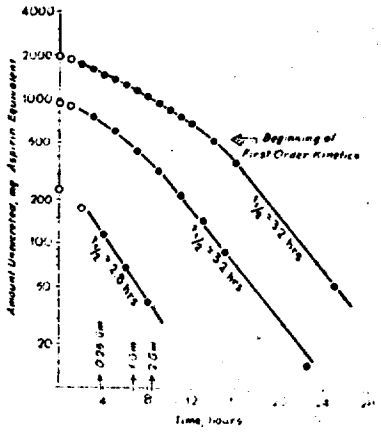


Fig. 2.—Elimination of salicylate by subject A as a function of dose. Doses taken were 0.25, 1.0, and 2.0 Gm. aspirin, respectively. Vertical arrows on the time axis indicate the time necessary to eliminate 50% of the dose.

A detailed tabulation of the data collected after administration of 1.5 and 2.0 Gm., respectively, of aspirin is presented as Tables IV and V. Not included in these tables are blank values obtained from the time of termination of drug elimination to about 70 hr. after drug administration. From the tables it can be seen that the average salicyluric acid output was about 78 mg. or 400 μm./hr. from the second to the eighth hour and 60 to 70 mg./hr. thereafter until elimination changed from zero to first-order kinetics. Salicylic acid excretion was essentially an exponential process, but exhibited the well known sensitivity to urinary pH (20). This can be seen in Table IV at 3 and 5 hr., when urinary pH and salicylate excretion rate were relatively high, and at 7¼, 8, and 10 hr., when urinary pH and salicylate excretion rate were relatively low. Similar effects are noticeable in Table V—namely, at 5, 9, and 16 hr. (high pH) and at 4, 7, and 12 hr. (low pH). Salicyl glucuronide elimination as listed represents both ester and ether glucuronides and, since the maximum excretion of the former occurs considerably earlier than that of the latter (21), there is no clearly recognizable pattern of excretion rate *versus* time. In addition, the excretion rate of salicyl glucuronides is affected by the variable excretion rate of unchanged salicylic acid, since salicylate excretion and glucuronide synthesis are parallel and therefore competing processes.

First-order rate constant for overall salicylate elimination as well as for the individual elimination processes can be obtained from experimental data collected after administration of 0.25 Gm. aspirin,

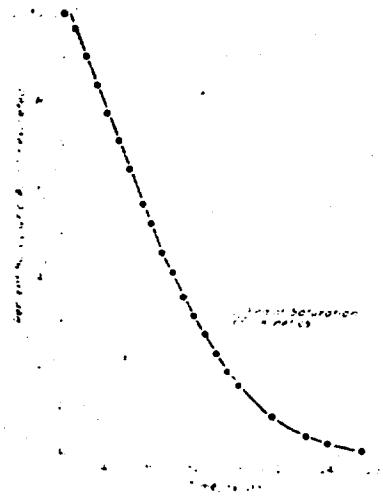


Fig. 3.—Excretion of salicyluric acid as a function of time after oral administration of 1.5 Gm. aspirin to subject B.

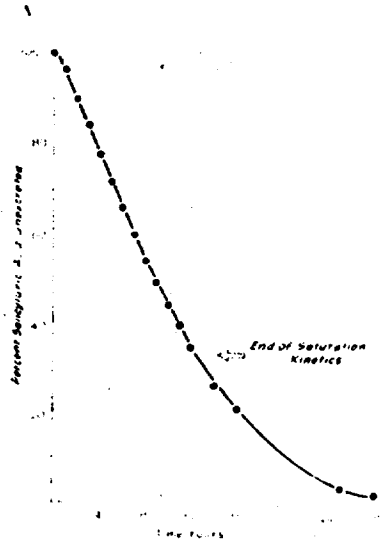


Fig. 4.—Excretion of salicyluric acid as a function of time after oral administration of 2.0 Gm. aspirin to subject A.

TABLE IV.—URINARY EXCRETION OF SALICYLATE AND ITS MAJOR METABOLITES AFTER ORAL ADMINISTRATION OF 1.5 GM. ASPIRIN (SUBJECT B)

Hr. After Drug Administration	pH	Salicylic Acid* Amt. Excreted, mg.	Salicylic Acid* Excretion Rate, mg./hr.	Salicyluric Acid* Amt. Excreted, mg.	Salicyluric Acid* Excretion Rate, mg./hr.	Salicyl Glucuronides* Amt. Excreted, mg.	Salicyl Glucuronides* Excretion Rate, mg./hr.	Cumulative Amt. of Total Salicylates Excreted, mg. Aspirin Equivalent
1	6.3	15.1	15.1	39.7	39.7	11.9	11.9	76
2	6.6	13.6	13.6	78.2	78.2	8.60	8.60	177
3	7.1	17.3	17.3	81.8	81.8	5.37	5.37	282
4	6.6	14.3	11.3	78.5	78.5	11.4	11.4	388
5	6.95	15.7	15.7	71.8	71.8	5.45	5.45	485
6	6.7	12.4	12.4	80.8	80.8	7.88	7.88	586
7½	5.7	2.47	1.98	96.8	77.4	12.9	10.3	695
8	5.9	2.38	3.17	55.0	73.3	6.58	8.77	778
9	6.1	1.71	1.71	69.9	69.9	8.82	8.82	849
10	5.5	0.99	0.99	64.4	64.4	7.04	7.04	916
11	5.65	1.29	1.29	69.2	69.2	8.21	8.21	986
12	5.8	1.56	1.56	53.3	53.3	6.18	6.18	1045
13	6.4	2.76	2.76	19.8	19.8	7.05	7.05	1104
14	6.9	5.75	5.75	55.1	55.1	5.05	5.05	1169
15	7.1	4.0	4.0	50.5	50.5	5.24	5.24	1227
16	5.6	0.45	0.45	37.5	37.5	5.15	5.15	1269
19	5.9	1.20	0.40	88.2	29.4	10.2	3.40	1356
22	5.5	0.55	0.18	19.8	16.6	10.2	3.40	1425
24	5.45	0.27	0.13	19.4	9.7	4.67	2.33	1450
27	6.6	0.83	0.28	17.4	5.8	5.25	1.75	1474
30	6.45	0.59	0.20	5.60	1.87	1.57	0.52	1482
32	5.3	0.13	0.06	2.25	1.12	0.87	0.43	1485
36	5.8	0.23	0.06	2.39	0.60	0.03	0.007	1488
39	6.5	0.29	0.10	0.59	0.20	0.82	0.27	1490

* Expressed in terms of salicylic acid

TABLE V.—URINARY EXCRETION OF SALICYLATE AND ITS MAJOR METABOLITES AFTER ORAL ADMINISTRATION OF 2 GM. ASPIRIN (SUBJECT A)

Hr. After Drug Administration	pH	Salicylic Acid* Amt. Excreted, mg.	Salicylic Acid* Excretion Rate, mg./hr.	Salicyluric Acid* Amt. Excreted, mg.	Salicyluric Acid* Excretion Rate, mg./hr.	Salicyl Glucuronides* Amt. Excreted, mg.	Salicyl Glucuronides* Excretion Rate, mg./hr.	Cumulative Amt. of Total Salicylates Excreted, mg. Aspirin Equivalent
1	7.0	39.6	39.6	45.8	45.8	18.9	18.9	118
2	7.05	30.9	30.9	81.5	81.5	8.85	8.85	245
3	6.6	17.7	17.7	74.8	74.8	15.3	15.3	358
4	6.25	9.93	9.93	81.3	81.3	19.5	19.5	471
5	6.9	23.7	23.7	77.3	77.3	15.7	15.7	594
6	6.6	17.3	17.3	71.5	71.5	15.6	15.6	705
7	6.25	10.1	10.1	74.1	74.1	19.5	19.5	812
8	6.5	15.4	15.4	72.2	72.2	20.1	20.1	925
9	7.13	21.6	21.6	60.6	60.6	14.6	14.6	1029
10	7.0	13.9	13.9	63.7	63.7	14.4	14.4	1124
11	6.6	6.45	6.45	59.3	59.3	14.6	14.6	1206
12	5.5	1.07	1.07	61.8	61.8	17.1	17.1	1287
14	6.7	15.6	7.80	107.7	53.8	26.5	13.2	1442
16	7.35	21.8	10.9	64.2	32.1	48.1	24.0	1592
25	6.35	14.0	1.55	225.9	25.1	67.2	7.47	1906
28	6.6	1.13	0.38	20.2	6.73	7.55	2.52	1936
31	6.35	0.86	0.29	8.2	2.73	2.40	0.80	1948
34	5.8	0.44	0.15	3.82	1.27	1.18	0.39	1954
37	5.8	0.36	0.12	1.85	0.62	0.49	0.16	1957
39	7.0	0.35	0.17	0.77	0.38	0.10	0.05	1958

* Expressed in terms of salicylic acid.

since elimination at this dose level is an exponential process. Thus,

$$\log A = \log A_0 - \frac{k}{2.3} t \qquad \text{(Eq. 1)}$$

where A is the amount of drug remaining unexcreted at time t, A_0 is the total amount of drug eventually excreted, and k is the first-order elimination rate constant, expressed in reciprocal hours. k can be determined from the slope of the straight line obtained when log A is plotted as a function of time. Half-life ($t_{1/2}$) values can be calculated on the basis of the relationship

$$t_{1/2} = \frac{0.693}{k} \qquad \text{(Eq. 2)}$$

The first-order rate constants for component processes can be calculated from k values and the frac-

TABLE VI.—KINETIC PARAMETERS FOR SALICYLATE ELIMINATION DETERMINED AFTER ADMINISTRATION OF 0.25 GM. ASPIRIN TO FOUR HUMANS

Subject[a]	$t_{1/2}$, hr.	k,[a] hr.$^{-1}$	k_{SU}, hr.$^{-1}$	k_{SA+SG}, hr.$^{-1}$
B	3.1	0.224	0.164	0.016
A	2.8	0.248	0.183	0.017
C	2.7	0.257	0.203	0.010
D	3.0	0.231	0.182	0.017
Mean[b]	2.9	0.239	0.182	0.032

[a] Ages and amounts excreted are listed in Table III. [b] $t_{1/2}$ expressed as arithmetic mean; rate constants as harmonic means.

tion of the total excreted dose which is eliminated as the particular metabolite, provided that rate of formation is considerably lower than rate of excretion (22). This is true for salicyl glucuronides and for salicyluric acid, as will be shown subsequently. Thus

$$k_{SU} = \frac{SU}{ST} \cdot k \qquad \text{(Eq. 3a)}$$

where k_{SU} is the first-order rate constant for salicyluric acid synthesis, SU is the total amount (in moles) of salicylate which is eliminated as salicyluric acid, ST is the total amount (in moles) of salicylate eliminated in all forms, and k is the first-order rate constant for over-all salicylate elimination. Similarly,

$$k_{SA+SG} = \frac{SA + SG}{ST} \cdot k \qquad \text{(Eq. 3b)}$$

where SA and SG are the total excreted amounts of salicylic acid and salicyl glucuronides, respectively. Separate estimation of k_{SA} and k_{SG} values is possible also (though not necessary for the purpose of the present study), but experimentally determined k_{SG} values tend to be too low and k_{SA} values too high since a considerable part of salicyl glucuronides may be hydrolyzed prior to urine collection (9). On the other hand, k_{SA+SG} will not be affected by partial hydrolysis of salicyl glucuronides. Values for k, $t_{1/2}$, k_{SU}, and k_{SA+SG} for four subjects are listed in Table VI. The mean half-life of salicylate elimination was found to be 2.9 hr., and the major process responsible for salicylate elimination at this low dose was the conjugation of salicylic acid with glycine to form salicyluric acid.

The renal excretion kinetics of salicyluric acid were determined by oral administration of this metabolite of salicylic acid. Salicyluric acid was well absorbed and rapidly excreted as shown in Fig. 5. Renal excretion was describable by first-order kinetics, showing no saturation effects in the dose range studied and no decrease in rate constant with increasing dose. The half-life for excretion was about 37 min., which is equivalent to a rate constant of about 1.1 hr.$^{-1}$. The maximum observed average excretion rate was 337 mg./hr., which is equivalent to 1730 μm., and thus is far in excess of the maximum excretion rate of salicyluric acid observed after salicylate administration (400 μm./hr.).

DISCUSSION

Kinetic Model for Salicylate Elimination.—The elimination of salicylate administered as aspirin can be depicted by the kinetic model in Scheme I.

In Scheme I, ASA is aspirin, SA is salicylic acid, SU is salicyluric acid, SG represents salicyl glucuronides, and the subscripts *b* and *u* refer to drug in the body and in the urine, respectively. k's are first-order rate constants for rate-limiting processes, while k^* designates first-order rate constants for processes that are not rate-limiting since they are preceded by processes with smaller rate constants (*i.e.*, $k_{SU} > k^*_{SU}$, and $k_{SG} > k^*_{SG}$). The formation of minor metabolites (such as gentisic acid) and excretion by extra-renal routes can be neglected in the following discussion, since these pathways are quantitatively insignificant.

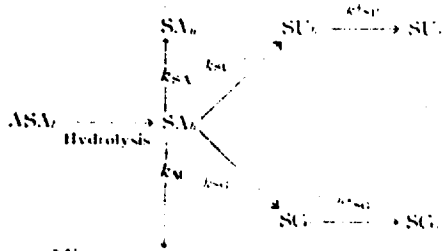
When 0.25 Gm. doses of aspirin are given, over-all salicylate elimination and therefore each of the participating processes proceeds by first-order kinetics. Thus

$$k = k_{SA} + k_{SU} + k_{SG} \qquad \text{(Eq. 4)}$$

where k is the first-order rate constant for over-all salicylate elimination. The *in vivo* hydrolysis of aspirin to salicylic acid is extremely rapid (23) as compared to salicylic acid elimination and thus need not be considered.

When higher doses of aspirin, such as 1 Gm. or more, are given, the formation of salicyluric acid reaches a maximum rate, and this process follows apparent zero-order kinetics until the amount of salicylate in the body has declined sufficiently for the formation rate to be lower than the maximum rate. The over-all elimination consists therefore of parallel zero- and first-order processes. This possibility has been suspected only recently (24, 25).

In the present investigation, the maximum salicyluric acid formation rate was found to be about 330 to 400 μm./hr. It is of interest that similar values can be found by examination of data previously reported by others. Schachter's data (9) indicate a maximal salicyluric acid excretion rate of 408 μm./hr. after administration of 4.2 Gm. sodium salicylate. Schachter and Manis found a maximal salicyluric acid excretion rate of 312 ± 66 μm./hr. in 19 subjects given 0.4 μm./Kg. of sodium salicylate (21). The data of Salassa and co-workers (26) indicate a maximum excretion rate of approximately 320 μm./hr. after administration of 3 Gm. sodium salicylate. Quick's maximum values, reported in 1933 (27), are somewhat lower (about 250 μm./hr. after ingestion of 1.0–3.5 Gm. salicylic acid), but this may be due to the less accurate analytical methods available at that time, or it may be a characteristic of the test subject used in his study. Clearly then, there has been indirect evidence in the



Minor metabolites and excretion by other than the renal route

Scheme I

literature for some time which should have suggested that salicyluric acid is formed at a constant low rate from salicylate even when the latter is administered in doses well within the therapeutic range. It is interesting that this has been overlooked for many years by workers in the field, including the author.

The limiting value for the excretion rate of salicyluric acid is not due to a renal tubular transport maximum [although salicyluric acid is known to be excreted by both glomerular filtration and active tubular secretion (28)]. This is evident from the exponential excretion of orally administered salicyluric acid even when excretion rate was far in excess of the maximum value observed after salicylate administration (Fig. 5).

It is known that the formation rate of hippuric acid by conjugation of benzoic acid with glycine also reaches a limiting value, but this maximum formation rate is considerably higher than the maximum rate of salicyluric acid synthesis. For instance, Quick (29) has reported maximum hippuric acid formation rates in the range of from 7300 to 9300 μm./hr. for an adult. Freiberg and West (30) have studied hippuric acid formation in children and found maximum rates about 50% higher (on a body weight basis) than those reported by Quick. Schachter (9) has reported that maximum hippuric acid formation rates in adults were around 7800 μm./hr., which is in agreement with the values established by Quick. Thus, it is evident that the maximum rate of conjugation of benzoic acid with glycine is about 20 times higher than the maximum rate of conjugation of salicylic acid with glycine. It will be shown in a subsequent report that this is due to the fact that the rate-limiting process in hippuric acid formation is not the same as the rate-limiting process in salicyluric acid formation (31).

Relationship Between Dose and Relative Rate of Elimination.—The kinetic model for salicylate elimination presented above and the kinetic parameters listed in Table VI make it possible to reconcile the wide differences in reported half-lives of salicylate elimination which are summarized in Table I. According to the findings of the present study, the half-life of salicylate elimination is about 2.9 hr. when the administered dose is sufficiently small so that all elimination processes proceed by first-order kinetics. This is in good agreement with the shortest reported half-life of salicylate elimination (Table I). When the administered dose is very high, the contribution of the zero-order process to salicylate elimination is negligible until most of the drug is eliminated. At these high doses, elimination occurs mainly by salicylate excretion and salicyl glucuronide formation. The rate constant for these combined processes was found in the present study to have a mean value of 0.032 hr.$^{-1}$ (Table VI), which is equivalent to a half-life of about 22 hr. This is similar to the maximum value reported by Done (12) in the case of salicylate poisoning, and it agrees also with the longest average half-life reported for salicylate elimination under therapeutic conditions, as tabulated in Table I. Thus, the relationship between dose and the time required for 50% elimination is as follows. (*a*) When the dose is sufficiently small so that all elimination processes proceed by first-order kinetics, the half-life is about 2.9 hr. (*b*) When the dose is so large that the contribution of the salicyluric acid formation process to the elimination of salicylate is negligible, the half-life increases to about 22 hr. Elimination appears to be a first order process (until most of the drug is eliminated) since the contribution of the zero-order process is too small to be noticeable except during the terminal phase of salicylate elimination. (*c*) When the administered dose is intermediate in size the zero order formation of salicyluric acid can be a significant contributory factor to the total elimination of salicylate. Apparent half-lives will increase continuously with dose in the range between the minimum and maximum values reported above. However, salicylate elimination definitely does not follow first-order kinetics in this range, although this can be overlooked easily. A graph of the logarithm of plasma salicylate concentration *versus* time (following administration of 1 Gm. or more of aspirin) usually looks linear for a period of time equal to more than one apparent half-life even though elimination proceeds by parallel zero- and first order processes. The same is true for urinary excretion curves (see Figs. 1 and 2). Interpretation of such graphs is complicated further by the initial absorption phase (after oral administration) or distribution phase (after intravenous administration). A downward bend in such curves is usually only apparent if elimination rate is followed for very long times (*i.e.*, over three orders of magnitude of uneliminated drug). In the case of plasma salicylate determinations, this would require intensive blood sampling on an around-the-clock basis. For practical purposes (such as for calculating appropriate dosage regimens), apparent half-life values obtained by drawing a best-fitting line through the initial part of the semilogarithmic plot (*i.e.*, to the point of 50% drug elimination) can be used. However, rate constants derived from such values are best referred to as functional rate constants to distinguish them from "true" first-order rate constants. The true first-order rate constant for salicylate elimination cannot be used for calculating salicylate dosage regimens in the usual therapeutic dose range.

Some comments are appropriate concerning the maximum half-life for salicylate elimination as estimated in the preceding paragraph. This value was derived from k_{SA+SG} values determined in the present study. k_{SA} is extremely sensitive to urinary pH and can increase more than tenfold when urinary pH is raised from below 6.0 to above 7.0 (20). Thus, it is reasonable to expect considerable inter- and intrasubject variation in k_{SA+SG} and therefore in maximum half-life of salicylate elimination. In the present study, k_{SA+SG} values ranged from 0.017 to 0.047 hr.$^{-1}$, which is equivalent to half-lives of 15 to 41 hr. Indeed, half-lives as long as 30 hr. for salicylate elimination after ingestion of large doses have been reported (4). On the other hand elimination will be considerably more rapid, due mainly to an increase in k_{SA}, when urine pH is increased by administration of alkalinizing agents such as sodium bicarbonate or Tris.

Estimation of Threshold for Apparent Saturation of the Salicyluric Acid Formation Process. Since the maximum rate of salicyluric acid synthesis in healthy adults is about 1001 μm./hr., it is possible to calculate the amount of salicylate in the body which must be exceeded before salicyluric acid formation changes from first order to zero order kinetics. At

below threshold levels

$$\frac{dSU}{dt} = k_{SU} \cdot SA_b \qquad \text{(Eq. 5)}$$

where dSU/dt is the rate of formation of salicyluric acid, k_{SU} is the first-order formation rate constant, and SA_b is the amount of salicylic acid in the body. Rearranging the equation to solve for SA_b, and introducing the values 400 μm./hr. for dSU/dt and 0.198 hr.$^{-1}$ for k_{SU} (from Table VI) yields

$$SA_b = \frac{400\ \mu m./hr.}{0.198\ hr.^{-1}} = 2000\ \mu m. \text{ or } 2\ mmoles$$

This is equivalent to 276 mg. of salicylic acid or 360 mg. of aspirin. Reference to Figs. 1 and 2 shows that this was indeed the amount of aspirin equivalent in the body at which the kinetics changed. Additional evidence in support of these estimations is available from the urinary excretion data reported by Cummings and Martin (11), which are summarized in Table II of the present paper. The per cent of a dose of aspirin excreted in 7.5 hr. was the same with doses of 0.16, 0.24, and 0.32 Gm., but decreased successively with doses of 0.64 and 0.97 Gm. The *dose* of aspirin necessary to obtain a body content equal to or exceeding 2 mmoles of salicylate depends in part on the rate of absorption of the drug. With conventional tablets, this dose should be between 400 and 500 mg. aspirin. In other words, the body content of salicylate at which salicyluric acid formation rate reaches a maximum in human adults is exceeded ordinarily when only two aspirin tablets are taken! Similar apparent saturation effects are known for elimination of only a few other drugs (notably, the elimination of ethanol and glycine conjugation of benzoic and *p*-aminobenzoic acids), and such saturation is encountered only at considerably higher doses.

The "Constant Infusion" Case—Independent Verification of the Kinetic Model.—Schachter and Manis (21) have determined plasma salicylate and salicyl conjugate levels as a function of time, after administration of 0.4 mmole/Kg. sodium salicylate to adult humans. They found that plasma salicylate levels declined from an average of 1782 μmoles/L. at 2 hr. to 467 μmoles/L. at 24 hr. after drug administration. On the other hand, plasma salicylurate levels remained constant during the entire period—namely, 9.8, 10.1, 9.5, and 10.5 μmoles/L. at 2, 4, 8, and 24 hr., respectively. Such plateau effects are obtained typically when a drug is infused at a constant rate and is eliminated by first-order kinetics. Schachter and Manis used a dose of salicylate which was large enough (according to the calculations presented in the preceding paragraph) to result in salicyluric acid formation at a constant rate for a considerable time (apparently more than 24 hr.). This is equivalent to a constant infusion case and permits the application of an equation (32) which relates infusion rate (R_0), apparent volume of distribution (V_d), and the first-order elimination rate constant (k) to the plateau plasma level (C_P) eventually obtained:

$$\frac{R_0}{V_d k} = C_P \qquad \text{(Eq. 6)}$$

Equation 7 may be solved for V_d by introducing a value of 10 μmoles/L. salicyluric acid for C_P (from

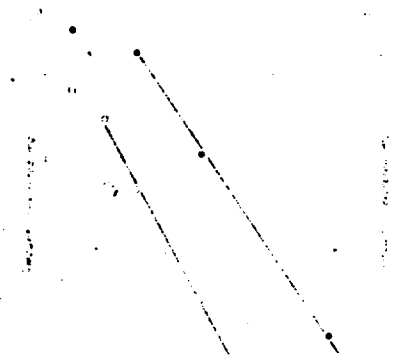


Fig. 5. Excretion of salicyluric acid as a function of time after oral administration of 0.1 Gm. (●) and 0.5 Gm. (○), respectively, of salicyluric acid (subject 1).

Schachter and Manis' data, 1.1 hr.$^{-1}$ for k (as determined in the present study from the experimental data shown in Fig. 5),[4] and 312 μmoles/hr. for R_0 (the value for the maximum rate of salicyluric acid formation, as found by Schachter and Manis). Thus

$$V_d = \frac{312\ \mu moles/hr.}{(10\ \mu moles/L.)(1.1\ hr.^{-1})} = 28.4\ L.$$

The renal clearance of a drug can be calculated from k and V since

$$\text{renal clearance} = \frac{kCV}{C} = kV. \qquad \text{(Eq. 7)}$$

where C is the plasma level of the drug. The renal clearance of salicyluric acid, as determined by Eq. 7 from a V_d value of 28.4 L. and k value of 1.1 hr.$^{-1}$ is 31.2 L./hr. or 520 ml./min. The renal clearance of salicyluric acid obtained experimentally by Schachter and Manis (21) was 444 ± 112 ml./min. The good agreement of these values constitutes an independent verification of the kinetic model for salicylate elimination developed in this paper and supports also the value for the first-order elimination rate constant for salicyluric acid obtained in the present study. The apparent volume of distribution of salicyluric acid determined above is two to three times greater than the volume of distribution of salicylic acid reported recently by Levy and Hollister (33), although exact comparison is not possible due to the concentration dependence of these values. However, the difference in volumes of distribution is consistent with the much greater plasma protein binding of salicylic acid, as compared to salicyluric acid (21).

Change in Salicylate Elimination Kinetics Due to Concomitant Administration of a Drug Which Competes for Glycine Conjugation. Following an initial observation by Dry *et al.* (34) that plasma salicylate levels increased when *p*-aminobenzoic

[4] In the context of the present discussion, k equals $(k_{SU} + k_U)$ as previously defined.

acid was co administered, Salassa *et al.* (26) studied the effect of *p*-aminobenzoic acid on the metabolism and excretion of salicylate. Their data (as depicted in Figs. 1 and 2 of their paper) show that elimination of salicylate (given as 3 Gm. sodium salicylate) proceeded at a practically constant rate (*i.e.*, by zero-order kinetics) and that most of the drug was eliminated as salicyluric acid. When sodium salicylate was given together with repeated doses of *p*-aminobenzoic acid (3 Gm. every 3 hr.), salicylate elimination occurred by first-order kinetics and there was practically no salicyluric acid formed. The half-life for salicylate elimination was about 26 hr. These observations can be rationalized and explained readily by means of the kinetic model and rate constants evolved in the present study. Salicylate elimination in the first experiment (without PAB) occurred primarily by salicylurate formation at a constant rate. The data show that this rate was about 320 μmoles/hr. which is in good agreement with the maximum rates observed in the present study. The over-all elimination process was describable by zero-order kinetics since the contribution of the first-order processes (salicylic acid excretion and salicyl glucuronide formation) was relatively small. When sodium salicylate was administered together with PAB, the latter competed so effectively with the former in the glycine conjugation process that there was almost no salicyluric acid formed. Under these circumstances, salicylate elimination was due almost solely to salicylic acid excretion and salicyl glucuronide formation. Since both of these are first-order processes, the over-all salicylate elimination proceeded by essentially first-order kinetics. The applicable rate constant for elimination under these conditions is k_{SA-SG}; and the corresponding mean half-life determined in the present study is 22 hr. (range 15 to 41 hr.). This agrees well with the 26-hr. half-life apparent from the data of Salassa and co-workers (26).

Salicylate Elimination Kinetics in the Newborn.—Earle (35) recently reported a case of congenital salicylate intoxication in an infant whose mother attempted suicide by taking 15–18 Gm. aspirin 27 hr. before the baby was born. The infant was treated by exchange transfusion, and plasma salicylate levels were determined subsequently for a total of 6 days. A plot of these values on a linear scale (rather than on a logarithmic scale as was done in the original paper) against time yields a straight line; *i.e.*, salicylate elimination apparently proceeded by zero order kinetics.[2]

Glucuronide conjugation capacity is deficient in the newborn (36). Blood level data obtained by West and Rossier (37) after administration of single doses of *N*-acetyl *p*-aminophenol (APAP) to newborn infants (1 to 6 days old) show a constant level of APAP glucuronide with time while APAP levels decreased. This is indicative of zero-order (saturation) kinetics with respect to phenolic glucuronide conjugation. Similar saturation conditions are probably operative in ester glucuronide formation. It is also known that renal function, particularly tubular function, is very poorly developed in newborn infants (38). Thus, salicylate elimination in newborns given appreciable amounts of the drug may be expected to involve mainly two parallel zero-order processes (salicyluric acid and salicyl glucuronide formation), with only a minor contribution from salicylic acid excretion processes. One of the latter (tubular secretion) is probably also saturated, while the other (glomerular filtration) may be expected to follow first-order kinetics. Under these conditions, over-all elimination will proceed at a very slow and constant (*i.e.*, zero-order) rate. This is evident from the data reported by Earle (35); plasma salicylate levels of the intoxicated infant declined at a constant rate and required over 70 hr. to decrease by 50%.

Additional Considerations.—Limitations in conjugating capacity, such as described with respect to the conjugation of salicylate with glycine, possibly may occur also with other drugs. For example, examination of recently reported blood levels of *p*-aminosalicylate obtained after administration of large single doses (39) suggests that zero-order kinetics may be operative in one or more of the elimination processes involved. A component zero-order process may be difficult to recognize on the basis of plasma level data in the presence of a parallel first-order process (or processes) of appreciable magnitude, but the occurrence of such kinetics should be considered whenever there is an apparent increased in half-life of elimination with increasing dose.[3]

Individual differences in apparent half-life of salicylate elimination, as reported by Levy and Hollister (2), probably reflect differences in maximum salicyluric acid formation capacity. Any search for genetic differences in salicylate elimination by salicyluric acid formation must be directed not only toward the determination of individual first-order rate constants for this process (which requires that the administered doses be small), but must also include the determination of individual maximum salicylurate formation rate capacities. Either one or the other (or both) could show genetically determined differences, if existent. An apparently genetically determined difference in glucuronide conjugation of salicylate and other drugs has already been observed (10).

When the observations of Alpen *et al.* (40), who studied the metabolism of salicylic acid in cancer patients, are interpreted against the background of information obtained or reviewed in the present study, the extent of salicyluric acid formation in these patients appears unusually limited. This raises the possibility that malignancy may decrease salicyluric acid forming capacity. It would be desirable to determine salicyluric acid forming capacity in children as a function of age, and to establish if this capacity is altered in arthritics and in other individuals taking large doses of salicylate for some time. There is presently no published information suggesting enzyme induction effects in the metabolism of salicylates. A recently reported study on dogs (41), which showed no change in salicylate elimination kinetics after repeated dosings

[2] This report was brought to the author's attention by Professor Eino Nelson, State University of New York at Buffalo, who also pointed out that the kinetics were apparently zero order.

[3] It has been reported recently (41) that the extent of acetylation of *p*-aminobenzoic acid (PABA) decreases with increasing dose, and increases when absorption rate is decreased. These observations suggest that acetylation of PABA occurs by apparent zero order kinetics when a certain body level of PABA is exceeded.

has only limited predictive value with respect to humans. This becomes apparent upon comparison of the human and dog data obtained by Salassa and co-workers (26).

ADDENDUM

Most biotransformation processes treated pharmacokinetically as apparent first-order reactions probably follow Michaelis-Menten kinetics (42, 43),

$$\frac{1}{v} = \frac{K_m}{V_{max.}C_s} + \frac{1}{V_{max.}} \qquad \text{(Eq. 1.1)}$$

where v is the initial velocity of the reaction, K_m is the Michaelis constant (which is numerically equal to the concentration of substrate necessary to attain half-maximum initial velocity), $V_{max.}$ is the maximum initial velocity (which is attained when the enzyme is "saturated"), and C_s is the concentration of substrate (*i.e.*, the drug). Equation 1.1 can be rearranged to

$$\frac{1}{v} = \frac{1}{V_{max.}}\left(\frac{K_m}{C_s} + 1\right) \qquad \text{(Eq. 2.1)}$$

to show that, when $K_m \gg C_s$, the expression reduces to

$$\frac{1}{v} = \frac{K_m}{V_{max.}C_s} \qquad \text{(Eq. 3.1)}$$

Equation 3.1, when inverted, yields the first-order rate equation

$$v = K\,C_s \qquad \text{(Eq. 4.1)}$$

where $K = V_{max.}/K_m$.

The maximum amount of a drug which can be administered safely to humans usually yields C_s levels which are very much smaller than K_m values. Therefore, the biotransformation process can be described adequately in these instances on the basis of first-order kinetics. While experimental difficulties (for example, control of the over-all process by another rate-limiting process) and toxicologic considerations have usually restricted the characterization of Michaelis-Menten kinetics of enzymic processes to isolated enzyme systems, cell fractions, or tissue slices, we have recently been able to demonstrate in intact man that salicylurate formation follows Michaelis-Menten kinetics (43).

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Proc. Soc. Exptl. Biol. Med. 6. 122-126, 1909

Some effects of sodium benzoate.

By DANIEL R. LUCAS. (By invitation.)

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This research was suggested to me by different experiences with sodium benzoate when taken by mouth in the following ways: A. Pure (1) as crystalline salt, or (2) in aqueous solution. B. In neutral or alkaline solutions, or in mixtures rich in fat, carbohydrate or protein, *e. g.*, milk. C. With vegetable or fruit acids (1) hot, as in tomato soup, or (2) cold, as in canned plums, oranges, lemons, etc. D. In beverages containing high percentages of organic acids, *e. g.*, cider, lemonade, grape juice, wine, etc. E. In mixtures containing inorganic acids, *e. g.*, artificial gastric juice.

Brunton has studied the effects of benzoic acid on enzymes and bacteria. The strong inhibiting effect of this substance on their activity is in striking contrast to the slight effect of sodium benzoate. Doepner has shown that fairly large quantities of sodium benzoate (2 per cent.) did not prevent the development of *Proteus vulgaris* and, in strengths equal to 0.5 per cent., only slightly retarded the development of *B. enteritidis*, *B. fluorescens* and *B. coli*. Fleck found that benzoic acid in concentrations equal to 0.6–0.7 per cent. caused marked inhibition of yeast fermentation and the inhibiting action was markedly decreased by the amount protein present. Lehman observed that meat extract putrefies in the presence of 1 to 2 per cent. of sodium benzoate, but less benzoic acid acts more strongly antiputrefactive when the reaction of the medium is markedly acid. The action of sodium benzoate under markedly acid conditions is the same as the action of benzoic acid. Under such conditions the action of the benzoate diminishes with decrease of

acidity. Chassevant and Garnier found that 1.4 gram of benzoic acid per kilo was fatal to guinea pigs in five to seven hours; larger doses (2 grams per kilo) did not necessarily kill sooner.

The results of my own work may be briefly summarized as follows:

Effects on microörganisms.—Sodium benzoate, in concentrations of about 1 per cent., preserves fruits and vegetables which are *strongly* acid. Crystals of free benzoic acid often appear in such mixtures. Sodium benzoate (1 per cent.) added to *weakly* acid fruits and vegetables does not preserve them well. Sodium benzoate (1 per cent.) added to fruits and vegetables, the acidity of which has been *neutralized*, does not preserve them. Pure apple juice, containing 0.1 per cent. of sodium benzoate, developed mould after ten days; commercial benzoated cider under the same conditions, without the further addition of benzoate, did not develop mould or otherwise undergo degeneration.

Effects on taste.—Acid fruit juices containing 1 per cent. of sodium benzoate, have a biting taste, an effect due to the liberated benzoic acid. Milk or alkaline vegetables treated with sodium benzoate (1 per cent.) do not taste of benzoic acid at any time during the first twenty-four hours after the treatment. After twenty-four hours, however, acid decomposition begins in milk in spite of the presence of 1 per cent. of sodium benzoate, when the mixture tastes distinctly of benzoic acid. Sips of 1 c.c. of orange juice, to which 1 per cent. of sodium benzoate has been added, cause burning in the posterior part of the mouth, the throat, the esophagus and stomach, with gastric discomfort, belching, uneasiness of the bowels and excessive passage of gas by rectum.

Experiments on men with cider. Pure cider.—Pure apple juice tastes sweet, bland; produces no stinging sensation in the throat; and is ordinarily enjoyed and well borne in volumes equal to 1,000 to 2,500 c.c. (ingested during a period of two or three hours). It is diuretic in action and, in amounts varying from 1,000 to 2,000 c.c., causes laxation of the bowels. This effect frequently depends on the rapidity with which it is ingested; it does not ordinarily cause laxation even when taken in large amounts, if ingested little by little. The average amount of pure apple juice consumed during an evening by adult males who

was able to ingest 1,000 c.c. of apple juice containing 0.5 per cent. of sodium benzoate, without any albuminuria arising. The amount of hippuric acid in the urine was very large for the first few hours. The secretion of urine was very much reduced for twelve hours, while I suffered from some of the other symptoms above mentioned, *although as a subject in a former investigation I ingested as much as 6 grams per day, for three successive days, in milk on a full stomach, without the slightest discomfort.*

Small doses of sodium benzoate given with *acid* substances to patients with albuminuria aggravated this condition and caused alarming symptoms, classical of nephritis—for six days thereafter in one subject.

Experiments on dogs. First experiment. — One dog weighing 3.5 kilos fasted for twenty four hours and was then given 1 gm. of sodium benzoate, decomposed with the theoretical amount of hydrochloric acid to form sodium chloride and free benzoic acid, with no excess of hydrochloric acid. In thirty minutes the animal showed evidences of muscular weakness and nausea, lay quietly and breathed in a laborious manner. This continued for six hours. On the next day, twenty four hours after the previous dose, the animal was given 4 gm. of sodium benzoate with a sufficient amount of hydrochloric acid to decompose it into benzoic acid, plus 120 c.c. of 0.2 per cent. citric acid. The animal became very weak in one hour, respirations were reduced to nine per minute, and were very labored. Tonic and clonic convulsions began one hour and fifteen minutes after the dose was given. The animal, after several hard convulsions, died two hours and twenty minutes after administration.

The autopsy showed congestion of various organs. There was very pronounced congestion of the kidneys, stomach and intestines, with ulceration in places. The liver and lungs also showed evidences of infarcts.

Second experiment. — Two dogs had been fed on dog biscuits and water for several weeks, and then fasted for thirty six hours.

Animal No. 1. — The first animal was a male, weighing 3.5 kilos. He was given a mixture of 3.5 gm. of sodium benzoate, 50 c.c. of water, 0.65 c.c. of concentrated hydrochloric acid (sp. gr. 1.19) and 100 c.c. of citric acid (0.2 per cent.). The animal

had free access to it was about 1,200 c.c. (twenty subjects). When from 1,000 to 2,500 c.c. of pure apple juice are consumed neither headache, nausea, albuminuria nor sub-normal temperature is produced. The specific gravity of the urine is greatly decreased when a liter of pure unfermented cider is consumed but the volume is markedly increased within forty-five minutes after its ingestion. The forty gallons of pure apple juice consumed by the human subjects of my investigation contained considerable apple pulp and 2.716 grams of free acid (calculated as acetic acid) per 1,000 c.c.

Benzoated cider. — Twenty four subjects were observed in the first experiment. Twelve received *pure* apple juice; twelve received samples of the same apple juice containing 0.1 per cent. of sodium benzoate. As none of the subjects knew that they were to receive at that time anything but pure apple juice, unfavorable psychological influences were eliminated from the experiments. Each subject received three question blanks to be filled out by himself daily as long as any symptoms lasted, which, I am assured, was done faithfully in every instance.

In comparison with those who received pure cider, the men who drank the benzoated apple juice exhibited the following special symptoms: Burning taste, fulness in the head, headache, nervousness, nausea, vomiting, itching of the skin, unusual perspiration, irregularity of bowels (*constipation* usually) *decreased* flow of urine, *increased* specific gravity of the urine, and albuminuria. Excessive amounts of hippuric acid were eliminated, especially during the first few hours, after ingestion of the benzoated apple juice.

Apple juice to which a small amount of sodium benzoate is added becomes sweeter to the taste, but astringent, stinging, and irritating to mucous membranes. The presence of 0.5 per cent. of sodium benzoate renders cider quite unpalatable, but the presence of 0.1 per cent. may be overlooked by subjects not acquainted with the taste of pure apple juice.

If the apple pulp is previously filtered from the juice the effects of added benzoate become much more evident. A liter of such filtered cider, containing 0.2 to 0.3 per cent. of sodium benzoate, caused albuminuria within three hours almost without exception in the largest and soundest picked subjects. However, I myself

became quite uneasy after receiving the dose. At the end of an hour he showed great muscular weakness and tremor.

Animal No. 2. — The weight of the second animal was 4.25 kilos. It was given a mixture of 100 c.c. of 0.2 per cent. citric acid, 50 c.c. of water and 0.85 c.c. of concentrated hydrochloric acid (sp. gr. 1.19). This animal was entirely unaffected.

The same experiments were repeated on the same animals the next day; the results were practically identical. The animal (1) that received the free acid-benzoic acid mixture, however, was more prostrated than on the previous day and showed general stiffness of the muscles. At the end of six hours it was chloroformed and autopsied, when it was found that the stomach contained "coffee ground" material. There were ecchymotic areas and some places appeared to be ulcerated slightly. The intestines showed marked congestion here and there and appeared to be slightly ulcerated in places throughout. The grumous material in the stomach and intestines gave a strong guaiac test and was undoubtedly modified blood. The liver and lungs showed considerable congestion with some evidences of infarcts. The kidneys were cyanotic, the cortex very much congested, while the medulla was pale and anemic.

Further investigation is contemplated, especially on the influence of nephrectomy on the toxicity of benzoic acid.

I am indebted to many members of the Purdue University Alumni Association of New York City for volunteering as subjects in this investigation and thus making it possible for me to carry out experiments on a large number of individuals. The Secretary, Mr. Leslie Hustable, Mr. Ray C. Ewry, Mr. R. W. Parks, Mr. F. M. Waltz and Mr. H. Worsham of that organization have given me special assistance in various ways. I am also indebted to Drs. A. E. Olpp and Matthew Steel, and Messrs. Herzfeld and Bisch for coöperation, and to Drs. Foster, Mosenthal and Rosenbloom for criticism and suggestions. Professor Gies has given me all the facilities of his laboratory for the conduct of this research, as well as valuable criticism and suggestions.

Arzneimittel-Forschung 10: 1033, 1960.

SHORT NOTES
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TOLERANCE FOR BENZOIC ACID BY PETER MARQUARDT

LANG was able to ascertain in rats experiments spread out over 4 generations with groups of 40 animals in each that no ill effects could be observed from daily intake in food of 1% benzoic acid, corresponding to 150 mg per diem, using as criteria growth, food utilization, lenght of life, reproduction, weight of organs. LANG, however, found that practically all animals died within a few weeks with 5% of benzoic acid added to food. **).

In this context we would like to report on experiments carried out in our Institute for 11/2 years in which 1.5% benzoic acid was fed.

We work with rats in individual cages. The animals were started in the experiment at an average weight of 50 - 60 gr., at an age of approximately 5 weeks. During the main period of growth, e.g. in the first 6 months, weight was controlled weekly, later every 4 weeks. The food consists of 66 % sugar, 32 % dried skimmed milk and 2 % dry liver. We, first, mix the benzoic acid with the dried milk powder, then add the dry liver and finally the powdered unrefined sugar. The animals receive water ad libitum. We use a Wistar strain which has been bred for years at the Surgical Clinic of Freiburg University by Prof. Dr. STUTZ. These experimental groups consist of 20 females and 30 males with controls of 12 females and 13 males. 15 animals of the experimental group and 3 controls so far have died. The weight difference between experimental animals and controls amounted to 10 gr. after 60 days, 18 gr.

**) W. Kiekebusch & K. Lang, Arzneim.-Forsch. 10, 1001 (1960)

after 340 days and 15 gr. after 430 days. The average weight of controls was 146.6 gr. on that day. Measured from the start of the experiment, the food intake difference amounted to 156.40 on the 375th day and 207.5 gr. on the 430th day.

The experiment has not yet been terminated.

Besides we also used rats from 2 other strains as random samples and with smaller totals. Both strains, a Wistar and a Osborne-Mendel strain were obtained from Prof. Dr. CREMER in Giessen, 20 males from each of the 2 strains were fed benzoic acid, 10 animals served as controls. The animals showed no difference in comparison with the controls, the weight difference between controls and experimental animals amounted to 30 gr. after 341 days for the Wistar strain with an average weight of 244 gr. for the controls. The difference in food intake was 335gr. up to that day. Similar observations were made on animals from the Osborne-Mendel strain. The weight difference amounted to 41 gr. on the 341,day with an average weight of the controls of 282 gr.. The difference in food intake was 70 gr.

At the present time, therefore, we can say that in all 3 groups the growth of animals fed with benzoic acid was slower than in the controls. Moreover, it was found that the food intake of animals fed benzoic acid is smaller than that of controls. The reason why we report these experiments briefly before their termination is that they already confirm and complement LANG's findings, that is that 1% benzoic acid added to food is the upper limit of tolerance.

METABOLISM OF BENZOIC ACID BY SHEEP

By A. K. MARTIN

Experiments have been made with two sheep to determine their ability to synthesise hippuric acid. The titratable acidity of diethyl ether extracts of acidified urine was expressed as g benzoic acid equivalent. One sheep on a dried grass ration and one on a hay and oats ration excreted in the urine 8·06 and 4·07 g benzoic acid equivalent/24 h, respectively, the amounts conjugated with glycine being 2·63 and 2·38 g respectively. When non-toxic levels of benzoic acid and glycine were infused into the abomasum, complete recovery of infused benzoic acid was obtained in terms of increased titratable acid in ether extracts of the urine. Infusion of 9·6 g of benzoic acid for 24 h as a continuous drip resulted in an increase of 11·89 g of benzoic acid equivalent in the urine, 8·90 g of which were conjugated with glycine. The largest non-toxic dose given was 53·95 g of benzoic acid which gave rise to an increase in urinary benzoic acid equivalent of 58·47 g, 27·10 g of which were conjugated with glycine. Lethal doses for the two sheep were 1·1 and 1·8 g of benzoic acid/kg body weight. Urine collected during infusion of a toxic dose to one sheep contained a large increment in combined glucuronides. A very large increase in urinary potassium was observed in one sheep given a toxic dose of benzoic acid.

Introduction

The enzymic formation of hippuric acid from glycine and benzoic acid is associated with the formation of AMP from ATP, that is with the consumption of two high-energy phosphate bonds per mole of hippuric acid synthesised.[1] It was thought that if a sheep was given benzoic acid in sufficient amounts, the energy cost of the synthesis could be measured by conventional methods of indirect calorimetry in the intact animal. The metabolisable energy required to form ATP from ADP and inorganic phosphate is[2] about 23 kcal/mole so the synthesis of 100 g of hippuric acid from glycine and benzoic acid would require about 26 kcal, that is, just within the limits of accuracy of calorimetric equipment when used with sheep.

Bray *et al.*[3] have reported that the rabbit can synthesise hippuric acid at rates of up to 480 mg/h or 11 g/day. This suggested that sheep might be able to synthesise sufficient hippurate for the present purpose. The experiments reported

here which were carried out as a preliminary to calorimetric work show, however, that the sheep has a more limited ability to produce hippuric acid from benzoic acid and glycine than has the rabbit, and that the amounts which can be formed are so small that any estimates of the energy cost of the synthesis in the intact animal would be highly inaccurate.

Experimental

Analytical methods

Aromatic acids in urine

In these experiments it was necessary to distinguish between urinary excretion of benzoic and hippuric acids. The method of hippuric acid determination previously used in this laboratory had been that of Hampton[4] which determines hippuric acid as the titratable acidity of ethyl acetate extracts of acidified urine. As benzoic acid and its homologues and also phenolic acids would be extracted under such conditions, such a method would clearly not be suitable in this experiment. Infusion of large amounts of benzoic acid in sheep would be expected to lead to excretion of free benzoic acid in urine if the animals capacity to synthesise hippuric acid were exceeded. It was decided therefore to determine hippuric acid in terms of the glycine content of acid hydrolysates of ether extracts of acidified urine.

Urine (20 ml acidified to pH 2–3) was extracted for 6 h in a continuous extraction apparatus, the ether distilled off and the extract hydrolysed under reflux for 3 h with 6 N-hydrochloric acid. The extract was neutralised with sodium hydroxide and the glycine content determined by the method of Alexander *et al.*[5] as modified by Smith.[6] Extraction of standard solutions of hippuric acid gave recoveries of 99·1–103·5% of the expected amount of glycine.

This method of determining urinary hippuric acid was compared with that of Hampton.[4] Eleven urine samples from sheep fed a variety of diets were collected over a 24 h period either into dilute hydrochloric acid (urine preservative) or as passed. Samples were analysed immediately after collection. Only 38–81% of the expected amounts of glycine were recovered assuming all the titratable acidic material to be due to hippuric acid. Storage of some of these urine samples for a further 24 h at 4° showed no change in the free glycine content or in that of the ether extracts. Since this work was completed, Nehring & Zelk[7] have reported that the titratable acidity of diethyl ether extracts of urine from cattle and pigs overestimates their hippuric acid content considerably.

The acids present in extracts prepared by each of the ether and ethyl acetate extraction were examined by paper chromatography using the n-butanol–ammonia–ammonium carbonate system of Fewster & Hall[8] in which the spots are made visible using a buffered methyl red spray at pH 7·0. Hippuric and benzoic acids have R_F values of 0·31 and 0·41, respectively, and, in all experiments, only two spots were found which corresponded to the movement of hippuric and benzoic acid standards. However, the apparent proportion of benzoic acid, as judged by the size of the spots was larger when the urine sample was extracted with ether than when it was extracted with ethyl acetate. It was thought possible that the temperature of extraction which was about 30° with ether extraction in the continuous extracter compared with room temperature (about 20°) in ethyl acetate extraction may have caused hydrolysis of hippuric acid during extraction. To test this, the same urine sample was extracted overnight in the continuous extraction apparatus with ethyl acetate. The temperature of the extraction column was about 70°. In this extract only a trace of hippuric acid was found on the paper chromatogram and a large benzoic acid spot.

The paper chromatograms after development according to Fewster & Hall[8] were sprayed with the *p*-dimethylaminobenzaldehyde reagent of Gaffney *et al.*[9] The orange-coloured hippuric acid azlactone was formed at the hippuric acid spot but the benzoic acid spot was no longer visible. Two other small spots were sometimes visible when this spray was applied, a yellow spot with R_F value of 0·34 and a pink spot with R_F value of 0·36. The yellow spot may have indicated the presence of traces of salicyluric acid, the presence of which has been reported in the milk and urine of cows by Booth *et al.*[10] Scott and co-workers[11] have reported that phenyl-substituted fatty acids in alkaline hydrolysates of sheep urine are in the ratio of approximately 75% benzoic to 25% phenylacetic with a trace of 3-phenylpropionic acid. The presence of phenylacetic acid would not readily have been detected with the paper chromatography system of Fewster & Hall[8] as its R_F value is very similar to that of benzoic acid.

In view of the partial hydrolysis of hippuric acid on continuous extraction, hippuric acid was determined as the increment in urinary glycine after hydrolysis for 3 h under reflux with 6 N-hydrochloric acid and the result expressed as g of benzoic acid conjugated with glycine. The total aromatic acid excretion was determined by acidifying urine to pH 2·5 and extracting for 6 h with diethyl ether. The ether was distilled off and the dry residue taken up in distilled water and titrated with sodium hydroxide. The result was expressed as g of 'benzoic acid equivalent'.

Glucuronic acids

Benzoic acid may also be excreted in urine conjugated with glucuronic acid and according to Quick[12] benzoyl glucuronide is very rapidly hydrolysed at high pH. The pH of the sheep urine samples collected in this experiment varied between 8·2 and 8·5, so some benzoic acid may have arisen from hydrolysis of benzoyl glucuronide. Free and combined glucuronides were estimated by the method of Fishman & Green.[13]

Other urinary analyses

Total *nitrogen* was determined by the Kjeldahl method using a copper sulphate and selenium catalyst. Urinary urea and ammonia were determined by the method of Van Slyke & Cullen.[14] Urinary potassium was determined in experiments with sheep O using an 'EEL' flame photometer.

Feed analyses

Nitrogen and crude fibre were determined by standard AOAC methods and lignin by the method described by Waite, Johnston & Armstrong.[15]

Experimental animals

Two wether sheep were used. Sheep A, which weighed 75 kg, was a seven-year-old Scottish Blackface and had a permanent cannula in the rumen. Sheep O, which weighed 47 kg, was a five-year-old Cheviot and had a permanent abomasal cannula.

Rations

Sheep A was fed 204 g of hay and 506 g of flaked maize (dry matter) per day. This low-roughage ration was low in benzoic acid precursors. Sheep O was fed 661 g (dry matter) per day of dried grass. This all-roughage ration was com-

paratively rich in benzoic acid precursors. Both rations were fed in two equal portions at 6 a.m. and 4.30 p.m.

Plan of experiments

The rations were fed to the sheep for 2 weeks before the start of the experiments. The sheep were then harnessed for urine collection as described by Wainman & Paterson.[16] The urinary excretions of the compounds or fractions described above were determined over four consecutive 24-h periods. Potassium was determined in the urine of sheep O only. No urine preservative was used and analyses were completed within 24 h of sampling. The average control excretion of each compound or fraction was taken as the mean of these four results. An experimental period for sheep O consisted of a 3- or 4-day period during which urine was collected and analysed as in the control period. During the first 24 h an approximately equimolar solution of glycine and sodium benzoate was infused through the fistula. The experimental period continued (except when toxic doses of benzoic acid were infused) until the daily urinary excretion of 'benzoic acid equivalent' and benzoic acid excreted as hippurate had returned to the control levels. Sheep A received only one infusion of 79·7 g of benzoic acid as sodium benzoate. Sheep O received four infusions (9·6, 22·4, 54·0 and 87·8 g) of benzoic acid as sodium salt. Between each experimental period a 4-day control period was run with the same urinary analyses as in the initial control period.

Results

Toxicity of benzoic acid

Sheep A given 79·7 g of benzoic acid in 24 h by rumen infusion, refused food offered 6 h after the infusion began. Urine was only collected for 48 h after the start of the infusion. Severe muscular weakness accompanied by muscular tremors developed on the second day after the infusion and respiration was rapid. The sheep died on the third day and at autopsy slight congestion of the abomasum and a fatty liver were noted.

When sheep O was given 9·6, 22·4 and 54·0 g benzoic acid in 24 h by abomasal infusion, no clinical signs were observed. When 88 g of benzoic acid were infused over 24 h, the sheep consumed a meal offered 6 h after the infusion started but refused that given at 20 h and died at the 24th hour. Severe muscular spasms and inco-ordination were noted.

Excretion of benzoic acid and its conjugation products when no additional benzoic acid was given

Table I gives the composition of the basal diets and the amounts of materials excreted in the urine when the basal diets were given. Sheep O given dried grass excreted almost twice as much 'benzoic acid equivalent' as sheep A but only 11% more 'benzoic acid equivalent' was conjugated with glycine. The amount of free glucuronic acid excreted by sheep O was slightly greater and that of conjugated glucuronic acids slightly less than the corresponding figures for sheep A. With each of the sheep, a considerable amount of 'benzoic acid equivalent' excreted was not conjugated with glycine. Some of this may have arisen from alkaline hydrolysis of benzoyl glucuronide. Assuming the whole of the free glucuronide arose by hydrolysis of benzoyl glucuronide, 38% of the total 'benzoic acid equivalent' was excreted unconjugated with glycine by sheep A and 64% by sheep O. In addition, if it be assumed that free glycine, which has been found to be stable in urine, arose from hydrolysis of hippuric acid before analysis, the unidentified 'benzoic acid equivalent' would be reduced to only 23 and 38% respectively of the total 'benzoic acid equivalent' excreted by sheep A and O respectively.

TABLE I

Intake of nitrogen and urinary excretion by sheep of some nitrogenous compounds and glucuronides. The ratio of 'benzoic acid equivalent' urinary excretion to crude fibre and lignin intake

Sheep	A	O
Diet	70% flaked maize 30% hay	Dried grass
Intake, g/day		
N	10·1	15·1
Crude fibre	73·0	187·9
Lignin	44·5	33·4
Excreted in urine, g/day		
Total N	5·57	9·80
Ammonia N	0·22	1·23
Urea N	3·22	5·91
'Benzoic acid equivalent'	4·07	8·06
Benzoic acid as hippurate	2·38	2·63
Free glucuronic acid	0·55	0·67
Combined glucuronic acid	1·07	0·54
Free glycine	0·18	0·94
'Benzoic acid equivalent' excreted per 100 g of lignin intake	9·2	24·1
'Benzoic acid equivalent' excreted per 100 g of crude fibre intake	5·6	4·3

Lignin is thought to be one of the principal precursors of aromatic acids in the urine of herbivores—see, for example, Csonka *et al.*[17] The sheep fed the dried grass diet however excreted 2·6 times as much urinary benzoic acid equivalent/100 g of lignin intake as the sheep fed the hay–maize diet. This was probably due to the greater maturity of lignin in the hay (which supplied 92% of the lignin of the hay–maize diet) made from grass cut between flowering and seed setting compared to that in dried grass which was cut prior to flowering. Pazur & DeLong[18] have reported that, as the maturity of red clover fed to sheep increases, so the urinary excretion of benzoic acid per 100 g of lignin intake falls. The ratio of urinary benzoic acid excretion to crude fibre intake was similar for both the hay–maize and dried grass diets.

Increments in urinary excretion of benzoic acid metabolites on infusion of benzoic acid and glycine

Table II gives details of the increases in urinary excretion of 'benzoic acid equivalent', hippuric acid, free and combined glucuronides and glycine following infusion of benzoic acid and glycine. These increases are the sum of each individual daily increase during the whole experimental period over the mean daily control output of each compound or fraction. It was possible to collect urine for only one day after the termination of the infusion to sheep A and for this reason recovery of 'benzoic acid equivalent' was incomplete for this animal.

In Table III the partition of incremental 'benzoic acid equivalent' between hippuric acid, benzoyl glucuronide and 'unidentified benzoic acid equivalent' is given, and in Table IV the corresponding increases in N-containing compounds. In each experiment the increase in nitrogen excreted in the urine was more than that given as glycine. This stimulation of

TABLE II

Infusion of benzoic acid and glycine to sheep during the experimental periods and the increase found in urinary excretion of aromatic acids etc. during this period over the mean daily excretion in the control period

Sheep	O	O	O	A
Infusion, g/day				
Benzoic acid	9·61	22·41	53·95	79·70
Glycine	6·14	14·32	33·44	49·44
Total increase in urinary excretion, g				
'Benzoic acid equivalent'	11·89	26·56	58·47	71·46
Benzoic acid as hippurate	8·90	14·63	27·10	23·43
Free glucuronic acid	*	*	0·43	12·60
Combined glucuronic acid	0·44	1·07	0·98	5·47
Free glycine	0·94	1·40	2·88	3·07

* No increase in free glucuronic acid found.

protein catabolism on dosage with benzoic acid has been frequently reported (see, for example, Ringer[19] and Arnstein & Neuberger[20]). As the amount of benzoic acid given increased, the percentage excreted as hippuric acid fell (Table III), and with the highest dose a large increase in free and combined glucuronides occurred. An increase in free glycine excretion occurred in each experiment, the maximum values being noted when the highest amounts were given. Urea- and ammonia-nitrogen made up the greater part of the increase in urinary nitrogen that occurred on infusion and which was not accounted for by hippuric acid and glycine.

Urinary loss of potassium for the experiments conducted with sheep O increased when benzoic acid was given. When 88 g, an acutely toxic dose, was given, the loss of potassium was 61 g per 24 h greater than the basal output. This was a very considerable depletion of body potassium.

Discussion

The results reported here show that as the amount of benzoic acid given was increased, a progressive fall occurred in the efficiency with which sheep conjugated benzoic acid and glycine. The death of one sheep 3 days after the infusion of 80 g of benzoic acid (1·1 g of benzoic acid/kg body-weight) and of the other 24 h after the infusion of 88 g (1·8 g/kg body-weight) indicates that 1 g of benzoic acid/kg body-weight is close to a lethal dose. Ringer[19] found that 1·3 g/kg body-weight was a lethal dose for the goat.

Kowalewski[21] has observed that dosing rats with sodium benzoate resulted in a 25% reduction in muscle potassium. The very large urinary loss of potassium following a toxic dose of benzoic acid given to sheep O confirms this result, and probably explains the muscular weakness observed when toxic doses of benzoic acid were given.

Das Gupta[22] reported that in Indian cattle benzoic acid was excreted completely conjugated with glycine and that any free benzoic acid found in the urine resulted from hydrolysis of hippuric acid after its excretion. If this were true, an amount of glycine equivalent to the benzoic acid hydrolysed would accumulate in the urine, since in control tests the glycine content of urine was found to remain unchanged over periods of up to 48 h after sampling. In the first three infusions to sheep O and that to sheep A, 3·0, 11·9, 32·4 and 40·1 g of benzoic acid infused were excreted as unidentified 'benzoic acid equivalent'. The glycine increment expected if this was free benzoic acid resulting from the hydrolysis of hippuric acid, would be 1·8, 7·3, 19·3 and 24·7 g respectively, whereas those found were only 0·9, 1·4, 2·9 and 3·1 g respectively.

These results suggest that sheep synthesise hippuric acid less efficiently than do rabbits. The maximum rate of hippuric acid synthesis found was with sheep O when 54 g of sodium benzoate were infused. It was 2·6 g/day/kg $W^{0.73}$ where kg $W^{0.73}$ is the metabolic body size. Assuming the

TABLE III

Increase in 'benzoic acid equivalent' excretion in urine following infusion of sodium benzoate and glycine and its partition between hippuric acid, benzoyl glucuronide and unidentified 'benzoic acid equivalent'

Sheep	Infusion of benzoic acid, g/day	Total increment of benzoic acid equivalent in urine, g	% Increment excreted as		
			Hippurate	Benzoyl glucuronide	Unidentified 'benzoic acid equivalent'*
O	9·61	11·89	73·2	2·2	25·6
O	22·41	26·56	53·8	2·4	43·8
O	53·95	58·47	45·9	1·5	52·7
A	79·70	71·46	31·3	15·1	53·6

* Increments in combined glucuronic acid are considered to represent benzoyl glucuronide, and in free glucuronic acid they are considered to represent hydrolysed benzoyl glucuronide; unidentified 'benzoic acid equivalent' has been calculated by difference.

TABLE IV

Amounts of glycine infused and the resulting increases in total nitrogen excreted and its partition between hippuric acid, glycine, ammonia and urea

Sheep	Glycine N infused, g/day	Increment in total urinary N, g	% Incremental N excreted as				
			Hippurate	Glycine	Ammonia	Urea	% Recovery of incremental N
O	1·15	5·89	17·0	2·9	15·3	68·4	103·6
O	2·67	7·39	26·8	3·5	14·5	45·1	89·9
O	6·24	8·97	33·8	6·0	25·3	10·5	75·6
A	9·22	10·03	17·7	6·4	12·5	47·4	84·0

rabbits used by Bray *et al.*[3] to have had a weight of 2 kg then the rates of synthesis they found varied between 3·9 and 7·0 g/day/kg $W^{0.73}$ when the rabbits were dosed with sodium benzoate and glycine.

Acknowledgments

The author thanks Dr. K. L. Blaxter for his interest and advice in the course of these experiments and Miss A. McLeay for analytical assistance.

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Received 25 March, 1966; amended manuscript 25 May, 1966

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Toxicol. Appl. Pharmacol. 19: 373, 1971.

30. *A Comparison of the Teratogenic Properties of Sodium Salicylate, Sodium Benzoate, and Phenol*, JAN L. MINOR and B. A. BECKER, College of Medicine, University of Iowa, Iowa City, Iowa.

The following sodium chloride controls and functional analogs of sodium salicylate were compared for teratogenic properties by ip administration to Sprague-Dawley rats on gestational (copulation evidence = day 1) days 9–11 or 12–14: sodium chloride, 600, 90 mg/kg; sodium salicylate, 500, 283, 158, 50 mg/kg; sodium benzoate, 1000, 315, 100 mg/kg; and phenol, 200, 63, 20 mg/kg. On days 12–14, fetal body weight was reduced from high control dose by high doses of all three drugs: 5.25 ± 0.17; 4.22 ± 0.06; 4.56 ± 0.05; 4.64 ± 0.08 g (respective to listing above). Gross anomalies were not observed. In utero deaths were increased by both sodium salicylate and sodium benzoate: 1, 26, 12, 1% (respective to listing above). On days 9–11 fetal body weight was reduced from control by sodium salicylate, ≧ 158 mg/kg, and sodium benzoate, 1000 mg/kg, but not by phenol: 5.10 ± 0.03, 3.98 ± 0.05, 4.75 ± 0.04, 4.12 ± 0.07, 5.09 ± 0.04 g, respectively. In utero deaths were increased by high doses of all of the sodium salts, but not by phenol; 15, 5, 99, 16, 12, 4%. Gross anomalies were observed with sodium salicylate, 500, 283, and sodium benzoate 1000 mg/kg. In conclusion, at equitoxic doses the teratogenicity appears to be associated with carboxyl moiety. The hydroxy group may contribute to, but is not necessary for, teratogenicity. (Supported by NIH Grant Nos. GM-12,675 and GM0141.)

Pharmacology 3: 1–5 (1970)

Combined Effects of Some Local Anesthetics with Sodium Benzoate

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The association of local anesthetics with other drugs has been used in order to increase their intensity and at the same time diminish the induction period of anesthesia.

Hazard et al. [1] have shown that diethylaminoethanol, p-amino benzoate, sodium benzoate and other compounds used in combination with procaine significantly increase the duration and intensity of its local anesthetic properties.

Hazard and *Montoya* [2], using the test of corneal and dental pulp anesthesia in the rabbit, have demonstrated that the effect of procaine can be restored if sodium benzoate is administered by intravenous injection when the anesthetic effect has terminated.

The present research was undertaken to study the changes induced by sodium benzoate upon corneal and dental pulp anesthesia in the rabbit.

Methods

Rabbits weighing from 2 to 3 kg were used for corneal and dental pulp anesthesia. The technique used for corneal anesthesia was that described by *Régnier* [3], as modified by *Lechat* [4].

The anesthesia was considered at its maximum when the animal was able to bear 200 stimuli every 5 min without showing the oculo-palpebral reflex.

The intensity of the anesthetic action was expressed as the total number of stimuli that the animal could bear on its cornea, from the administration of the anesthetic solution until the reappearence of the oculo-palpebral reflex.

The drugs and concentration used are indicated in the results.

Received: February 2, 1969.

The results are expressed as the mean of 10 experiments.

In order to test infiltrative anesthesia, two lateral cavities on the rabbits upper incisors one day prior to the experiment, were drilled 1 mm from the gum. The cavities were protected with Zinc oxide and Eugenol until the day of the experiment.

Electrodes which were connected to a neurostimulator, were placed in the cavities.

The electrical stimulation produced a response characterized by trismus alternated with masticatory movements followed by lapping. The intensity of the response varied according to the voltage employed.

The local anesthetics were administered in the gum of the rabbit.

Increasing voltages at a frecuency of 30 cps were used as stimuli, each lasting 10 msec for a period of 1 sec according to the method described by *Laffargue* [5].

The drugs used in dental pulp anesthesia were the same as those employed in the other experiments, but at different concentrations (see table II).

Results obtained from the dental pulp experiments have been expressed as the mean area of a polygon that results from relating the increase in threshold to time, as described by *Winter* and *Flataker* [6].

Results and Discussion

When a 1% solution of sodium benzoate was added to procaine, cocaine, lidocaine, tetracaine or dibucaine a significant increase in both intensity and duration and a decrease in the induction period of the anesthesia were observed. This synergism between local anesthetics and sodium benzoate was greater for procaine, cocaine and lidocaine than for tetracaine and dibucaine.

These results do not agree with the results of *Charonnat* [7] which show that sodium benzoate decreases the effect of the anesthetic. However, his investigation was carried out under different experimental conditions, namely the use of different concentrations of anesthetic drugs.

On the other hand, *Régnier* and *Quevauviller* [8] have established the hypothesis that the increase or decrease of permeability to an anesthetic solution should depend upon the salt used. Sodium benzoate should facilitate the passage of the anesthetic through the corneal membrane thus allowing a faster and longer action.

On Table II we have summarized the results obtained with the same anesthetic drugs employed alone and with sodium benzoate in the dental pulp of the rabbit. Our findings show that procaine

TABLE I

Effect of some local anesthetics administered either alone or combined with sodium benzoate upon the rabbit cornea

Drug	Concentration %	Anesthetic used alone		Anesthetic with sodium benzoate	
		Intensity[1] Mean ± S. E.	Duration[2] Mean ± S. E.	Intensity[1] Mean ± S. E.	Duration[2] Mean ± S. E.
Procaine	1.00	275 ± 29	17 ± 2	1990 ± 124[3]	46 ± 3
Cocaine	0.50	432 ± 79	15 ± 1	1554 ± 38[3]	36 ± 2
Lidocaine	1.00	910 ± 113	24 ± 2	1759 ± 87[3]	39 ± 1
Tetracaine	0.25	999 ± 125	26 ± 2	1776 ± 165[4]	44 ± 4
Dibucaine	0.05	763 ± 10	28 ± 2	1936 ± 307[4]	60 ± 4

[1] No. of stimulations failing to produce the oculo palpebral reflex.
[2] Minutes.
[3] $P < 0.001$.
[4] $P < 0.01$.

TABLE II

Effect of some local anesthetics administered either alone or combined with sodium benzoate upon the rabbit dental pulp

Drug	Concentration	Anesthetic used alone		Anesthetic with sodium benzoate	
		Intensity[1] Mean ± S. E.	Duration[2] Mean ± S. E.	Intensity[1] Mean ± S. E.	Duration[2] Mean ± S. E.
Procaine	2.00	29 ± 3	35 ± 2	62 ± 13	35 ± 2
Cocaine	1.00	10 ± 5	22 ± 2	10 ± 5	25 ± 2
Lidocaine	2.00	82 ± 16	38 ± 3	71 ± 17	40 ± 6
Tetracaine	0.50	82 ± 19	21 ± 1	52 ± 10[3]	32 ± 1[3]
Dibucaine	0.10	4 ± 2	21 ± 1	9 ± 4	24 ± 1

[1] Surface determined by a curve relating the increase in threshold to time in minutes.
[2] Minutes.
[3] $P < 0.001$.
[4] $P < 0.01$.

has a greater action when used with sodium benzoate, cocaine, lidocaine and dibucaine are not potentiated when used with sodium benzoate in infiltrative anesthesia. Furthermore, the anesthetic effect of tetracaine decreases when combined with sodium benzoate.

While sodium benzoate facilitated the passage of the anesthetic used through the corneal membrane, it did not have the same effect when these drugs were injected into the gum. In the latter case it might be supposed that other factors, such as a variation of the pH of the different solutions, determine a potentiation only for procaine but not for the other drugs used.

TABLE III

Effect of sodium benzoate upon the pH of some solutions of local anesthetic. Solutions employed in corneal anesthesia

Drug	Concentration %	Anesthetic used alone pH	Anesthetic with sodium benzoate pH
Procaine	1.00	5.85	6.55
Cocaine	0.50	4.90	6.40
Lidocaine	1.00	6.20	6.35
Tetracaine	0.25	5.60	6.45
Dibucaine	0.05	5.70	6.45

TABLE IV

Effect of sodium benzoate upon the pH of some solutions of local anesthetic. Solutions employed in dental pulp anesthesia

Drug	Concentration %	Anesthetic used alone pH	Anesthetic with sodium benzoate pH
Procaine	2.00	5.85	6.60
Cocaine	1.00	4.20	6.35
Lidocaine	2.00	6.20	6.30
Tetracaine	0.50	5.60	6.40
Dibucaine	0.10	5.90	6.75

The pH of the sodium benzoate-anesthetic solutions employed in the cornea and the dental pulp, are listed in tables III and IV. The effect of pH variations due to different concentrations of sodium benzoate will be the matter of a future investigation.

Summary

In the present work the effect of the simultaneous administration of sodium benzoate with procaine, lidocaine, cocaine, tetracaine or dibucaine in surface anesthesia (rabbit cornea) and infiltrative anesthesia (rabbit tooth pulp) was studied. Sodium benzoate decreased the induction time and increased the potency and the duration of the effect of all the anesthetics to a significant extent. This increase was greater for procaine, lidocaine, and cocaine than for tetracaine and dibucaine (rabbit cornea).

These results were compared with those obtained using the method of tooth pulp stimulation in the rabbit.

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CHROM. 4219

CHROMATOGRAPHY OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS AND COLUMNS

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(First received April 14th, 1969; revised manuscript received June 2nd, 1969)

SUMMARY

The separation of eight kinds of food preservative by one- and two-dimensional chromatography on polyamide layers was investigated. A comparison was made between the detection limits on polyamide and silica gel layers, and the superiority of the polyamide thin layers was confirmed.

The separation of the food preservatives on a polyamide column with two types of elution system was established. The recovery of these compounds was satisfactory.

INTRODUCTION

After establishing the efficacy of polyamide in chromatography[1-7], it has been applied extensively to column and thin-layer chromatography where it was found that the separation was very much influenced by the hydrogen bond formed between the HO-group in phenolic compounds and the -CONH-group in polyamide[1,2,8].

This paper deals with the separation of food preservatives (aromatic acids with and without a phenolic HO-group and its alkyl esters, and some other conventional preservatives) by column and thin-layer chromatography using polyamide as the chromatographic medium.

EXPERIMENTAL

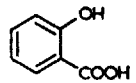
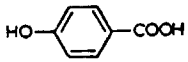
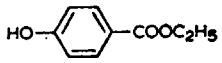
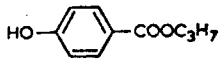
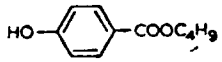
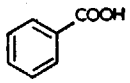
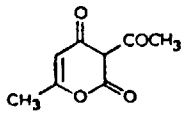
Materials

Polyamide powder from two sources, Polyamide Woelm TLC and Wakō Polyamide B-10* were used for thin-layer chromatography while for the column chromatography only Polyamide Woelm was used.

* "Wakō Polyamide B-10" contains 10% anhydrous calcium sulfate as a binder (Wakō Pure Chemical Industries, Ltd., Nihonbashi-honcho, Chuō-ku, Tokyo, Japan).

TABLE I

WAVE LENGTHS AND MOLAR ABSORBANCY INDICES OF FOOD PRESERVATIVES AT THEIR UV ABSORPTION MAXIMA

Sample		*λmax (mμ) and ε × 10⁻³*					
		In water		*In 1% (w/w) NH_3*		*In H_2O–AcOH–MeOH (5.5:0.5:4, v/v)*	
Salicylic acid		232 298	6.66 3.37			305[a]	3.60[a]
p-Hydroxybenzoic acid		251	11.8	280	17.5	256	15.0
Ethyl *p*-hydroxybenzoate		256	15.0	297	23.4	256	15.9
n-Propyl *p*-hydroxybenzoate		256	15.0	297	24.1	256	15.9
n-Butyl *p*-hydroxybenzoate		256	15.5	297	24.2	256	15.9
Benzoic acid		227.5	9.18			—[b]	—[b]
Sorbic acid	$CH_3—CH=CH·CH=CH·COOH$	260	23.6			262	29.3
Dehydroacetic acid		227.5 306	14.5 12.1			308	14.1

[a] λ max and its molar absorbancy of salicylic acid were measured in H_2O–AcOH–MeOH (3:1:6).
[b] λ max and its molar absorbancy of benzoic acid could not be measured in this medium.

The food preservatives used in this experiment are of a specially refined grade. The chemical and optical characteristics of the preservatives are as listed in Table I.

Preparation of the polyamide chromatoplates

(a) One gram of Polyamide Woelm TLC was mixed with 12 ml of ethanol to give a homogeneous suspension, which was spread evenly with a suitable applicator on a glass plate (20 × 20 cm) to a thickness of 0.25 mm. The plate was dried in air at room temperature and stored in a desiccator.

(b) Fourteen grams of Wakō Polyamide B-10 were made into a homogeneous suspension by mixing it with 56 ml of distilled water. The chromatoplate was prepared in the same way as (a).

Solvent systems for thin-layer chromatography

The solvent systems (A–F) used in the experiments are listed in Table II.

Application of samples and development of chromatoplates

For one-dimensional chromatography, 0.5 μl of an 0.2 % (w/v) ethanolic solution of each preservative was applied except for dehydroacetic acid and benzoic acid where 1.5 μl of a sample of the same concentration was used. Sample application was made on a line 3 cm from the lower edge, and the sample was developed by the ascending method.

In order to ensure the equilibration of the vapor in the chamber, the inside was lined with filter paper soaked in the solvent.

The development was continued until the solvent reached 12 cm from the point of sample application.

For two-dimensional chromatography, 1 μl of an 0.2 % (w/v) ethanolic solution of the samples was applied; as above increased amounts (2 μl) of dehydroacetic acid and benzoic acid were applied. Samples were spotted at a point 3 cm from both edges.

Detection of the spots on the chromatoplates

The spots can be seen under UV light as dark areas against a bright background, but for confirmation's sake *p*-hydroxybenzoic acid, its esters, and salicylic acid were detected as reddish violet spots by spraying with diazotized sulfanilic acid reagent; and dehydroacetic acid, sorbic acid and benzoic acid gave reddish spots against a yellow background with Methyl Red reagent.

The composition of the reagents used are as follows:

Diazotized sulfanilic acid. One gram of sulfanilic acid, dissolved in 8 ml of conc. HCl and 100 ml of distilled water, is added to the same volume of 0.09 % $NaNO_2$ solution.

Methyl Red. A mixture of 10 ml of 0.1 % ethanolic solution of Methyl Red and 20 ml of 0.1 *M* phosphate buffer (pH 7.0).

Preparation of the polyamide column and application of samples

Forty-five grams of Polyamide Woelm were washed with distilled water, methanol and chloroform, successively. After drying in air, the washed polyamide powder was suspended in the same solvent as that used for elution, and the powder was packed into a glass column (1.7 × 50 cm) to form a column bed of 45 cm height. The samples

TABLE II

SOLVENT SYSTEMS FOR CHROMATOGRAPHY OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS

Symbol	*Components*	*Ratio (v/v)*
A	MeOH–H_2O	6:4
B	Acetone–H_2O	5:5
C	MeOH–AcOH–H_2O	5:1:4
D	MeOH–conc. NH_3–H_2O	2:1:7
E	AcOH–H_2O	3:7
F	*n*-Hexane–AcOH	8:2

TABLE III

R_F VALUES OF FOOD PRESERVATIVES ON POLYAMIDE LAYERS

(*a*) R_F *values on polyamide* (*Woelm*)

Sample	*Solvent system*					
	A	*B*	*C*	*D*	*E*	*F*
Salicylic acid	0.05	0.05	0.78	0.56	0.14	0.25
p-Hydroxybenzoic acid	0.13	0.18	0.30	0.96	0.16	0.04
Ethyl *p*-hydroxybenzoate	0.32	0.41	0.38	0.64	0.16	0.11
n-Propyl *p*-hydroxybenzoate	0.27	0.37	0.32	0.55	0.10	0.15
n-Butyl *p*-hydroxybenzoate	0.22	0.32	0.29	0.44	0.07	0.19
Benzoic acid	0.14	0.21	0.46	0.85	0.23	0.56
Sorbic acid	0.20	0.29	0.56	0.92	0.32	0.64
Dehydroacetic acid	0.18	0.25	0.73	0.79	0.54	0.45

(*b*) R_F *values on polyamide* (*Wakō B-10*)

Sample	*Solvent system*					
	A	*B*	*C*	*D*	*E*	*F*
Salicylic acid	0.12	0.16	0.25	0.75	0.29	0.40
p-Hydroxybenzoic acid	0.31	0.34	0.53	0.95	0.33	0.06
Ethyl *p*-hydroxybenzoate	0.53	0.63	0.61	0.76	0.34	0.15
n-Propyl *p*-hydroxybenzoate	0.49	0.58	0.56	0.68	0.25	0.21
n-Butyl *p*-hydroxybenzoate	0.44	0.54	0.52	0.58	0.18	0.28
Benzoic acid	0.34	0.33	0.66	0.91	0.43	0.62
Sorbic acid	0.43	0.44	0.72	0.93	0.55	0.68
Dehydroacetic acid	0.45	0.40	0.83	0.86	0.73	0.50

(each 10 μmoles) were dissolved in 5 ml of the same solvent and applied on the top of the column. The elution was performed at a flow rate of 0.22–0.25 ml per min, and each 3.5 ml fraction was collected. The concentration of the food preservatives in the fractions was determined by measuring the absorbancy at its λ max* in the medium used for elution.

* In the case of benzoic acid, the absorbancy was measured at 240 mμ, as benzoic acid has no absorption maximum in H_2O–AcOH–MeOH (5.5:0.5:4).

RESULTS AND DISCUSSION

Thin-layer chromatography

The solvent systems suitable for the separation of the food preservatives on polyamide layers are summarized in Table II. They are classified into two types, solvents A–E and solvent F, according to the polarity of the constituent solvents. Tables III a and b show the R_F values of the food preservatives in the above solvent systems.

It may generally be concluded from these tables that the Woelm Polyamide possesses stronger adsorption properties than the Wakō Polyamide B-10.

Hydrogen bonding effects can be observed from the R_F values of salicylic acid and *p*-hydroxybenzoic acid which are known to form an intra-molecular hydrogen bond and inter-molecular hydrogen bond, respectively.

Comparison of the differences in the R_F values of these compounds in any two types of solvent A–E or one of these and solvent F shows that a complete separation could be obtained by two-dimensional development in either of the following combinations:

Solvent B–Solvent F
Solvent B–Solvent E

Of all the possible combinations of the solvent systems listed in Table II, these two combinations gave the best distribution of spots on the chromatograms (Fig. 1).

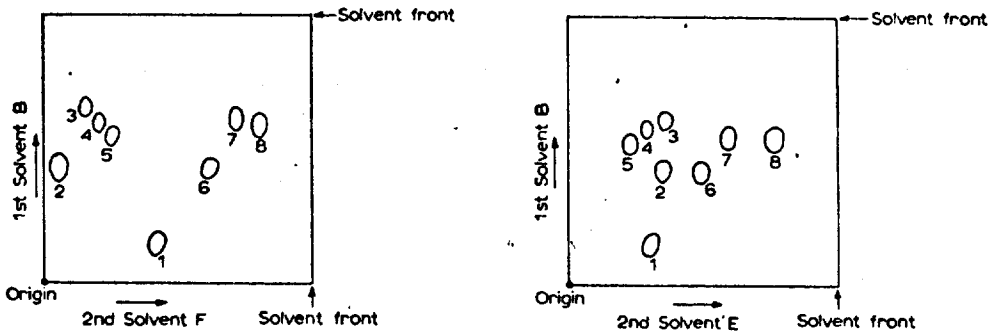


Fig. 1. Two-dimensional chromatograms of food preservatives on polyamide layers. 1 = salicylic acid; 2 = *p*-hydroxybenzoic acid; 3 = ethyl *p*-hydroxybenzoate; 4 = *n*-propyl *p*-hydroxybenzoate; 5 = *n*-butyl *p*-hydroxybenzoate; 6 = benzoic acid; 7 = sorbic acid; 8 = dehydroacetic acid.

One of the characteristics of the polyamide thin layers is to give a bright background in transmitted UV light, and this phenomenon provides a highly sensitive method of detection of UV-absorbing substances on the chromatoplate[9,10]. As shown in Table I, all the food preservatives tested absorb the UV light, thus they are all detectable as dark spots under transmitted UV light. In Table IV, a comparison is made between the detection limit of the spots on polyamide layers and on silica gel layers. It can be seen from Table IV that the detection of these substances is more sensitive on polyamide layers than on silica gel layers. With regard to other methods of detection of the spots, the Methyl Red reagent and the diazotized sulfanilic acid reagent can be used on the polyamide layers as well as on silica gel layers (see EXPERIMENTAL).

TABLE IV

DETECTION LIMITS OF FOOD PRESERVATIVES BY UV ABSORPTION ON POLYAMIDE LAYERS AND SILICA GEL LAYERS

Sample	*Polyamide in µg*	*Silica gel*[a] *in µg*
Salicylic acid	0.1	0.5
p-Hydroxybenzoic acid	0.25	5–10
Ethyl *p*-hydroxybenzoate	0.25	5–10
n-Propyl *p*-hydroxybenzoate	0.25	5–10
n-Butyl *p*-hydroxybenzoate	0.25	5–10
Benzoic acid	3[b]	50[b]
Sorbic acid	0.25	5–10
Dehydroacetic acid	2[b]	25–50[b]

[a] Fluka Silica gel (Fluka AG, Chemische Fabrik, Switzerland) was used.

[b] The low sensitivities of both these compounds are due to the large discrepancy between their absorption maxima and the wave length (2537 Å) of the UV light (see Table I).

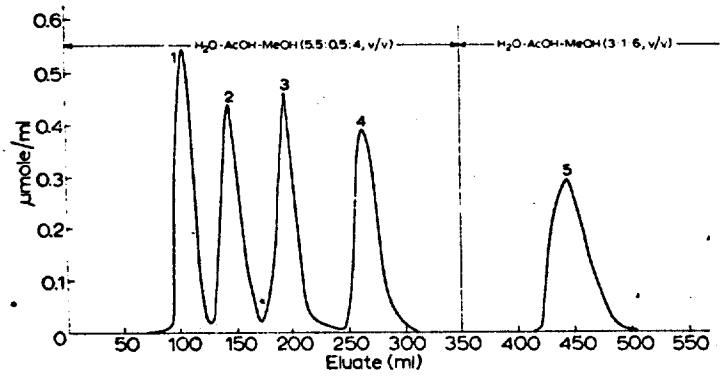


Fig. 2. Separation of food preservatives by polyamide column chromatography. Samples: 1 = dehydroacetic acid; 2 = sorbic acid; 3 = benzoic acid; 4 = *p*-hydroxybenzoic acid; 5 = salicylic acid; each 10 µmoles applied. Column: Polyamide Woelm for column use; 1.7 × 45 cm.

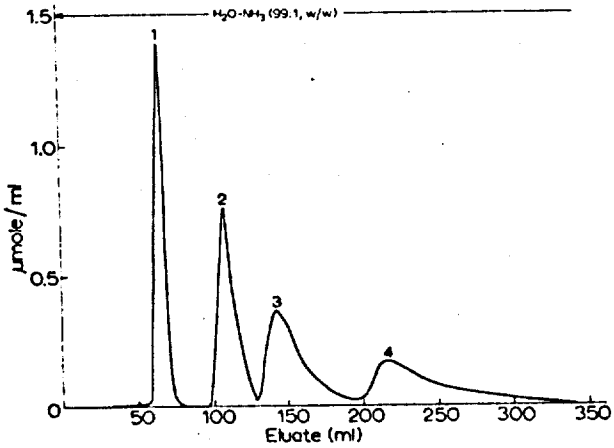


Fig. 3. Separation of food preservatives by polyamide column chromatography. Samples: 1 = *p*-hydroxybenzoic acid; 2 = ethyl *p*-hydroxybenzoate; 3 = *n*-propyl *p*-hydroxybenzoate; 4 = *n*-butyl *p*-hydroxybenzoate; each 10 µmoles applied. Column: Polyamide Woelm for column use; 1.7 × 45 cm.

Column chromatography

Suitable conditions for the separation of the food preservatives on column chromatography have been established as shown in Figs. 2 and 3. A mixture of salicylic acid, *p*-hydroxybenzoic acid, benzoic acid, sorbic acid and dehydroacetic acid was separated on a polyamide column by stepwise elution using two elution systems: (1) H_2O–AcOH–MeOH (5.5:0.5:4) and (2) H_2O–AcOH–MeOH (3:1:6) successively (Fig. 2). The recovery of the samples in this separation system was satisfactory, as is shown: dehydroacetic acid 95.5 %, sorbic acid 96.5 %, benzoic acid 101.2 %, *p*-hydroxybenzoic acid 100.1 %, salicylic acid 103.2 %. An elution procedure using water containing NH_3 (1 %, w/w) was selected for the separation of a mixture of *p*-hydroxybenzoic acid and its alkyl esters. Separation was attained as shown in Fig. 3 and recovery was also good: *p*-hydroxybenzoic acid 92.0 %, ethyl *p*-hydroxybenzoate 96.0 %, *n*-propyl *p*-hydroxybenzoate 98.3 %, *n*-butyl *p*-hydroxybenzoate 91.0 %.

Because neither of the elution media above used has any absorption in the UV, the concentration of each preservative in the fractions was directly determined by measuring the absorbance at the λ max (see Table I).

CONCLUSION

Eight kinds of food preservative, which include five phenolic hydroxyl compounds, one enolic hydroxyl compound and two carboxylic compounds, were separated on polyamide layers. From the correlation of the mobility of the samples and the solvents used, it was noticed that some hydrogen bonding forces have a definite effect on the interaction between samples having phenolic hydroxyl groups and the polyamide molecule, and that the polarity of the solvents exerts a considerable influence on the hydrogen bonding. The detection of the spots on the polyamide layers by the UV absorption method is 5–20 times more sensitive than for those on silica gel layers. This confirms that polyamide layer chromatography is suitable for the micro-analysis of food preservatives.

The conditions for the separation of these substances on a polyamide column were established. The simultaneous separation of all the samples was not possible, but by combining two types of elution system, *viz.*, a H_2O–AcOH–MeOH system and a H_2O–NH_3 system, good separations in two groups of samples were attained. The recovery of the samples separated on the polyamide column was also satisfactory.

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THE CONJUGATION OF BENZOIC ACID IN MAN*

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(Received for publication, April 2, 1931)

The extensive investigations on the conjugation of benzoic acid, since Wöhler's discovery in 1824 of the synthesis of hippuric acid by the animal, have failed to furnish a comprehensive and definite concept of the metabolic and chemical changes taking place when benzoic acid is introduced into the organism. No attempt has ever been made to explain why man eliminates benzoic acid almost completely as hippuric acid while the dog excretes it mainly as glycuronic acid monobenzoate. Indeed many investigators have entirely ignored the fact that benzoic acid can be and is combined with glycuronic acid, and have recorded their determinations of total combined benzoic acid as hippuric acid. The seriousness of this error can be readily appreciated when one recalls that in the dog, glycuronic acid monobenzoate constitutes roughly three-quarters of the total combined benzoic acid, and that even in man varying amounts of benzoic acid combined with glycuronic acid may be excreted. The lack of a specific and reliable quantitative method until recently for the determination of hippuric acid has been another serious source of error.

The conjugation of benzoic acid constitutes a problem which deserves careful study because it offers a promising approach to the investigation of intermediary metabolism. The well known theory of β-oxidation of fatty acids was developed from the study of the fate of benzoic acid and its homologs in the body. It is highly probable that further work on the behavior of aromatic compounds, such as benzoic acid, which can elicit a physiological

* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

response without themselves becoming oxidized and completely destroyed, may furnish further significant facts that bear on the problem of the intermediary metabolism of both carbohydrates and protein. With this object in mind the present research was undertaken. It was realized however that before much progress could be made, it was essential to know more about the basic factors that influence the absorption, conjugation, and elimination of benzoic acid in both man and animals. Since the human and the dog differ most in their handling of benzoic acid, the work will be limited for the most part to these two species, and the present report deals exclusively with the problem of benzoic acid in man.

HISTORICAL

Normal Excretion of Hippuric Acid

The literature on benzoic acid is too extensive to allow a complete bibliography. Many papers, furthermore, are concerned with the fate of benzoic acid in the lower animals and have little bearing on the problem of the formation of hippuric acid in the human.

The earliest work was confined to the qualitative determination of hippuric acid following the ingestion of various substances. It was found that besides benzoic acid, other compounds, notably, benzaldehyde (1), cinnamic acid (2), toluene (3), and quinic acid (4), gave rise to an increased output of hippuric acid. Lücke (5) observed that the excretion of hippuric acid was also elevated after the ingestion of vegetables and fruits, especially cranberries. Blatherwick and Long (6) have found that a man may produce as much as 10 gm. of hippuric acid after eating 450 gm. of prunes and 4.7 gm. after consuming 305 gm. of cranberries. It is commonly accepted that quinic acid is the substance present in fruit which is responsible for the increased excretion of hippuric acid. The amount of preformed benzoic acid in fruits is too small to account for all of the hippuric acid. Blatherwick and Long found only 0.147 and 0.096 per cent respectively in prunes and cranberries. The benzoic acid from this source constitutes therefore only 6 per cent of the total amount excreted after eating prunes. Recently Kohman and Sanborn (7) have stated that they have isolated quinic acid from prunes and cranberries.

Salkowski (8) and Baumann (9) pointed out the possibility of benzoic acid originating from the bacterial decomposition of protein. The small amounts of hippuric acid that are excreted during fasting or on a diet restricted as to vegetables and fruit are undoubtedly due to the abnormal metabolism of amino acid containing the benzene nucleus, but this problem has never been intensively studied.

Toxicity of Benzoic Acid

The toxicity of benzoic acid is even now a mooted question. Wiley (10) stated emphatically that sodium benzoate even in small doses (0.9 to 2.5 gm.) frequently caused toxic symptoms especially nausea and vomiting, and concluded that benzoic acid added to food was injurious to health. The Referee Board of Consulting Experts composed of Chittenden, Long, and Herter (11), on the other hand, could find no deleterious effects from moderate doses (up to 4.0 gm. daily). The results reported by others are equally as contradictory. Meissner and Shepard (12) found that in certain individuals 5.7 gm. of sodium benzoate produced marked gastrointestinal disturbance, whereas Lewinski (13) and more recently Bignami (14) found individuals who could tolerate 25 to 40 gm. with no or only minor symptoms. Sodium benzoate has even been given therapeutically in doses as high as 12 gm. daily without ill effects (15).

The toxic symptoms appear to be of two kinds: first, those arising from the local irritation of the gastrointestinal mucosa. The common symptoms are nausea, vomiting, diarrhea, which is sometimes even bloody, and anorexia. The second type of disturbance arises presumably from the action of benzoic acid on the central nervous system. In man this is manifested as headache, tinnitus, vertigo, giddiness, and reflex vomiting. The more severe symptoms such as convulsions and respiratory failure have been observed only in animals.

Source of Glycine

Many of the recent papers on benzoic acid have been devoted to a study of the synthesis of glycine, and naturally most of these investigations have been carried out on lower animals. A good review of the literature has been given by Griffith and Lewis (16)

and by Csonka (17). The more important facts at least tentatively established are: (1) The glycine employed for the synthesis of hippuric acid is partially synthesized, and partially obtained from the food. (2) Addition of glycine or of a protein rich in glycine accelerates the rate of hippuric acid excretion in the rabbit (16), increases the output of hippuric acid in the pig (17, 18), and to a less degree in the dog (19). Kingsbury (20) reported that he encountered two healthy individuals with subnormal output of hippuric acid following the ingestion of sodium benzoate, whose excretion became normal when glycine was added to the diet. (3) Glycocholic acid probably is not a precursor of glycine, and even it were, the question of the original source of glycine for glycocholic acid would not be answered. (4) The effect of benzoic acid on nitrogen metabolism is still incompletely understood. Much of the experimental evidence is of doubtful value because the hippuric acid is calculated from the total combined benzoic acid and no account is taken of the presence of glycuronic acid monobenzoate. Several important studies have been carried out on man. Lewis (21) found that after taking sodium benzoate a decrease in the excretion of urea and ammonia resulted. Shiple and Sherwin (22) made similar observations and further noted that the benzoic acid did not cause an increase in the total nitrogen output. Lewis and Karr (23) recorded a definite decrease in the excretion of uric acid which made them suspect that this substance might be the precursor of glycine. Swanson (24), however, found a concomitant increase in the blood uric acid but no increase in the urea. He concluded that glycine is probably synthesized from the constituents which are normally converted to urea. Bignami (14) from his experiments of feeding excessively large doses of sodium benzoate concluded that the body had 13 gm. of glycine and 4.0 gm. of glycuronic acid available for conjugation.

Site of Synthesis of Hippuric Acid

Since the classical work of Bunge and Schmiedeberg (25), the kidney has been considered the organ in which the conjugation of benzoic acid with glycine takes place. Snapper, Grunbaum, and Neuberg (26) have repeated these early experiments of perfusing the kidney of dogs with benzoic acid and glycine and have corrobo-

rated the older findings. They also found a similar synthesis taking place in the kidney of other species, and they made the remarkable and important observation that even the human kidney when perfused with glycine and benzoic acid synthesizes hippuric acid. Whether the kidney is the only organ which possesses this power is questioned by some authors. Kingsbury and Bell (27) recorded actual isolation of hippuric acid in the blood of double nephrectomized dogs, and Friedmann and Tachau (28) isolated hippuric acid after perfusing the liver of a rabbit with blood containing benzoic acid but no glycine. These authors could find no glycine in the liver, and they arrived at the significant conclusion that the glycine is synthesized in the liver in response to benzoic acid.

Attempts to use the conjugation of benzoic acid with glycine as a clinical test have not met with much success. The literature has been carefully reviewed by Bryan (29). The most important finding is the delayed excretion of hippuric acid in the type of nephritis which is characterized by a retention of nitrogenous substances, but there is no indication that the actual synthesis of hippuric acid is impaired. In fact Snapper and Grunbaum (30) found an increased concentration of hippuric acid in the blood stream after feeding benzoic acid to patients with contracted kidneys. Bryan believes that the excretion of free benzoic acid is an indication of liver impairment and accounts for it as an inability of the liver to mobilize glycine. Unfortunately no cognizance was taken of the possible presence of glycuronic acid monobenzoate which is readily hydrolyzed and can thus give rise to much free benzoic acid.

Excretion of Glycuronic Acid Monobenzoate

Senator (15) in 1879 noted a strong reducing substance in the urine of patients who were taking large doses of sodium benzoate. Later Kobert (31) considered the appearance of a reducing substance in the urine as a valuable sign of sodium benzoate intoxication. Lewinski (13) also noted that the urine of individuals who took large doses of sodium benzoate had reducing properties and was dextrorotatory. Magnus-Levy (32) in 1907 showed that the reducing substance was a compound of benzoic acid combined with glycuronic acid. Csonka (17) in 1921 studied the quantitative relationship of benzoic acid conjugated with glycine and with glycuronic acid in the pig. In 1926, the writer (33) isolated the compound in pure crystalline form, studied its chemistry, and renamed it glycuronic acid monobenzoate.

It is generally considered that in man small doses of benzoic acid are quantitatively excreted as hippuric acid. Dakin (34) found only slight reduction in the urine of men who took 10 gm. of benzoic acid. Neuberg (35), however, found definite evidence that even when as small a dose as 5.0 gm. of benzoic acid is taken, a portion may be conjugated with glycuronic acid, and that the difference between the total combined benzoic acid and hippuric acid may be as great as 7 to 12 per cent. The writer (36) has found that when glycuronic acid monobenzoate is ingested in moderate amounts, none of it is excreted in the urine, but the output of hippuric acid is definitely increased. From this he concluded that the body can oxidize and utilize conjugated glycuronic acid, and that the amount of glycuronic acid appearing in the urine is not a measure of the amount synthesized, but rather of how much has escaped oxidation.

EXPERIMENTAL

Most of the data were obtained on the same subject, but the more important experiments were repeated on two other individuals. Sodium benzoate[1] was taken with 200 cc. of water in the morning immediately on rising, and no food was eaten until the experiment was completed. It was found, however, that a breakfast consisting of toast and coffee did not affect the results. The urine was collected hourly, and preserved with toluene.

Analytical Methods

Hippuric Acid—The method described by the author (19) was used. Because of the high concentrations of hippuric acid encountered in this work, 5 cc. samples were usually employed for analysis. After the hydrolysis of the hippuric acid with hydrochloric acid, and subsequent evaporation to dryness, the residue

[1] Benzoic acid was always ingested as sodium benzoate and never as the free acid. In this paper, however, all values for benzoic acid are expressed as such and not as sodium benzoate, in order to maintain a uniform and convenient system for recording and comparing quantitative data.

was dissolved in hot water, treated with decolorizing charcoal, and the filtrate analyzed by the formol titration method directly without diluting to a definite volume as originally proposed. The method was again critically checked, and a typical result on a sample of human urine which contained no glycuronic acid monobenzoate nor free benzoic acid was as follows:

By the method of Kingsbury and Swanson (37)..... 2.20 gm.
" " author's method..... 2.17 "

Glycuronic acid monobenzoate was determined by the Shaffer-Hartmann method as previously described, free benzoic acid by the

TABLE I

Excretion of Hippuric Acid after Eating Quinic Acid and Prunes

Time	Benzoic acid excreted as hippuric acid	Remarks
hrs.	*gm.*	
0–4	0.16	6.0 gm. quinic acid taken
4–8	0.48	
8–12	0.60	
12–22	1.18	
22–27	0.26	
0–3	0.15	250 gm. prunes taken
3–13	1.25	
13–17	0.43	
0–8	1.01	500 gm. prunes taken
8–16	1.95	
16–24	0.90	

method of Raiziss and Dubin (38). Uric acid was determined by the Morris and Macleod method (39) with use, however, of Benedict's reagent (40).

DISCUSSION

Quinic Acid as a Precursor of Benzoic Acid

A study of the rate of hippuric acid elimination following the ingestion of prunes gives indirect evidence that the source of benzoic acid is quinic acid. As will be seen later, the excretion of ingested benzoic acid is very rapid, whereas the benzoic acid de-

rived from quinic acid is eliminated very slowly (see Table I). When 6 gm. of quinic acid were taken *per os*, only 0.15 gm. of benzoic acid was excreted during the first 4 hours. After that the rate gradually increased but the elimination was not complete even after 24 hours. When prunes were eaten similar results were obtained. There was at first a pronounced delay in the excretion of hippuric acid, followed by a slow but gradual increase in the rate and a prolonged period of excretion. It is interesting that Meissner and Shepard in 1866 noted qualitatively this prolonged excretion of hippuric acid following the ingestion of quinic acid. The cause for this delay is not known. It is unlikely that it is due to the slow absorption of quinic acid from the intestinal tract. In one experiment the prunes exerted a violent laxative effect which resulted in an apparent complete elimination of the prune residue in 4 hours; nevertheless, the excretion of hippuric acid continued for over 14 hours. It appears probable that the elimination of hippuric acid represents the rate with which quinic acid is reduced in the body to benzoic acid. It should also be stated that quinic acid may not exist free in fruits but is perhaps combined as a glucoside.

Glycine, as an Important Factor in the Synthesis of Hippuric Acid

The absorption of sodium benzoate from the intestines appears to be very rapid, and probably is many times faster than the excretion of hippuric acid. Therefore, after the first 30 to 60 minutes following the ingestion of the drug, the rate of absorption need not be considered a major factor limiting the synthesis and excretion of hippuric acid. In animals such as the rabbit and the dog, the rate of hippuric acid elimination is essentially the same whether sodium benzoate is given *per os* or parenterally, and it seems reasonable to assume that similar results could be obtained on humans.

The fundamental factor governing and limiting the formation of hippuric acid is the rate of the synthesis of glycine by the body. It can readily be seen by inspecting Chart I that the body does not have a store of preformed glycine. Benzoic acid itself appears to be the stimulus for the synthesis of this amino acid; the greater the quantity of benzoic acid administered, the greater the amount of glycine produced until the maximum capacity for this synthesis

is reached. Thus, by the simple experiment of feeding sodium benzoate one can determine not only the rate at which glycine is formed, but also ascertain the maximum quantity of glycine which can be produced per unit of time. Again referring to Chart I, attention is called to the fact that no matter how large the amount of benzoic acid administered, the hourly excretion of hippuric acid rises to a maximum and then remains constant until all but a small portion is eliminated. That this fixation of the excretion of hippuric acid is entirely due to a limitation of the synthesis of

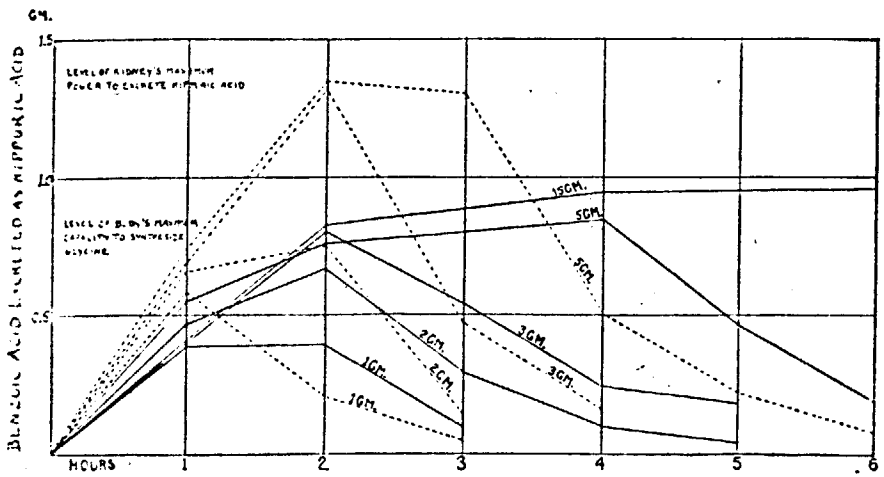

CHART I. The excretion of hippuric acid following the ingestion of benzoic acid in the fasting state, and when glycine is fed. The solid line indicates benzoic acid alone administered; the broken line, benzoic acid with glycine.

glycine can be conclusively demonstrated, for on the administration of glycine the hourly excretion of hippuric acid is promptly and decidedly increased (see Tables II to IV).

It has generally been accepted that the body can build up glycine, but the fact that the maximum rate of this synthesis can be definitely determined seems to have hitherto been overlooked. From the limited number of individuals studied, it was determined that the body can produce from 0.55 to 0.70 gm. of glycine per hour, the amount depending roughly on the surface area of the body. It is interesting to note here that Bignami (14) re-

ported that when he fed 20 to 40 gm. of benzoic acid to fasting men, only 21 gm. of benzoic acid appeared conjugated as hippuric

TABLE II

Excretion of Hippuric Acid after Ingestion of Benzoic Acid and of Hippuric Acid, with and without Glycine

Subject Q. Weight, 52 kilos; height, 164 cm.; surface area, 1.55 sq. m.

	Benzoic acid excreted as hippuric acid										
Benzoic acid taken....	1 gm.		2 gm.		3 gm.		5 gm.			Hippuric acid, 7.5 gm.*	
Time		With glycine†		With glycine†		With glycine†		‡	With glycine§		With glycine
hr.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.38	0.58	0.46	0.66	0.39	0.69	0.55	0.74	0.74	0.84	0.84
2	0.39	0.21	0.66	0.75	0.80	1.32	0.76	0.81	1.35	1.37	1.38
3	0.10	0.05	0.29	0.14	0.54	0.47	0.80	0.77	1.30	0.82	0.82
4			0.10		0.24	0.16	0.84	0.91	0.51	0.36	0.38
5			0.04		0.18		0.47		0.22	0.23	
6							0.19		0.08		

* Equivalent to 5.0 gm. of benzoic acid.
† 2.0 gm. of glycine.
‡ 0.5 gm. of theobromine taken at the beginning of the experiment.
§ 3.0 gm. of glycine.

TABLE III

Benzoic Acid Excreted as Hippuric Acid

Subject S. Weight, 58.0 kilos; height, 172 cm.; surface area, 1.70 sq. m.

Time	Urine volume	*	With glycine†	
			Urine volume	*
hr.	*cc.*	*gm.*	*cc.*	*gm.*
1	90	0.55	350	1.12
2	200	0.96	575	1.61
3	175	1.03	200	0.95
4	80	1.01	90	0.31
5	35	0.33	49	0.06
6	25	0.14		

* Took 5.0 gm. of benzoic acid.
† 3.0 gm. of glycine.

acid in 24 hours. This is equivalent to 13 gm. of glycine, a figure which agrees admirably with those found in the present investigation.

It is rather surprising that the potential capacity of the body to produce glycine should be so great. It is somewhat difficult to compare this synthetic power of man with that of the lower animals since very little data are available. From results reported by Griffith (41) it was calculated that the rate of the synthesis of glycine in the rabbit is about 0.025 gm. per hour per kilo of body weight. Similar calculations of Csonka's results (17) indicate that for the pig it is 0.015 gm. and from unpublished data it was determined that for the dog the rate is 0.0035 gm. per hour. On the basis of the data recorded in Table II, the rate of synthesis of glycine in man is approximately 0.009 gm. per hour per kilo of body weight. Thus, both the rabbit and the pig possess a relatively greater power to synthesize this amino acid than man, whereas the dog is distinctly limited in his ability to produce glycine.

TABLE IV

Benzoic Acid Excreted as Hippuric Acid

Subject W. Weight, 77.5 kilos; height, 188 cm.; surface area, 2.02 sq. m.

Time	Urine volume	*	With glycine†	
			Urine volume	*
hr.	*cc.*	*gm.*	*cc.*	*gm.*
1	120	0.85	130	1.22
2	99	1.11	130	1.84
3	72	0.63	130	0.80
4	53	0.61	130	0.30

* Took 5.0 gm. of benzoic acid.
† 3.0 gm. of glycine.

Why the organism should possess such an efficient synthetic mechanism is rather difficult to understand. In the new born the necessity of the body to synthesize glycine is very evident, for this substance is needed in large quantities for growth, whereas milk, the sole food of the suckling, contains practically no glycine. It hardly seems probable however that this anabolic process has merely been carried over from early life. Glycine undoubtedly plays a more active physiological rôle than is generally appreciated. Obviously, a considerable quantity must be produced to form glycocholic acid, and it is suggestive that a

relationship exists between the dog's limited power to synthesize glycine and the absence or exceedingly low content of glycocholic acid in the bile of this animal. An interesting problem arises whether it is possible to change the relative proportions of glycocholic and taurocholic acid by either depriving the animal of glycine, which can be accomplished by feeding large quantities of benzoic acid, or by feeding excessively large amounts of glycine.

It seems quite probable that the prolonged feeding of benzoic acid can produce profound physiological disturbances which depend not primarily on the toxicity of benzoic acid, but rather on the diversion of glycine from normal metabolic channels. Thus, Griffith's results (42) on feeding sodium benzoate to growing rats kept on a diet low in glycine can perhaps be explained by the assumption that the capacity of the rat to synthesize glycine was sufficient to conjugate the benzoic acid but inadequate to supply the normal requirements. The beneficial results which he later obtained (43) by adding glycocholic acid to the diet are apt to depend more on the fact that the organism is relieved from the stress of furnishing glycine to produce this needed bile acid rather than that the latter compound acts as a precursor of glycine.

Another possible function of glycine must be considered. Brühl (44) from microscopic studies of the glomerular circulation of the frog has observed that a lack of glycine stops the circulation in the glomerulus. Similar observations were recorded earlier by Watzadse (45). In the light of these findings, an explanation may perhaps be found for the increased output of uric acid when glycine is fed as was observed by Lewis, Dunn, and Doisy (46), and recently corroborated by Christman and Mosier (47). Similarly, an answer may be found for the question why benzoic acid causes a sudden and marked drop in the excretion of uric acid.

Amino acids, of which glycine is no doubt the most available, seem to exert a direct stimulating effect on the kidney which manifests itself in an increase in the rate of excretion of uric acid. Amino acids can therefore almost be considered hormones to the kidney. Presumably, when the supply of exogenous amino acids is exhausted, glycine continues to be synthesized so that the excretion of uric acid proceeds normally. If, however, benzoic acid is administered, all of the glycine is diverted to be conjugated with the benzoic acid, thus producing an acute deficiency of glycine.

In the kidney this result produces a marked decrease in the excretion of uric acid, and as Swanson has shown, a piling up of the uric acid in the blood. If this hypothesis is correct, the drop in uric acid excretion should be prevented by supplying the organism with sufficient glycine before giving sodium benzoate. That this can actually be accomplished is seen by inspecting Table V. Instead of getting the usual decrease in uric acid excretion after giving sodium benzoate, an actual increase was effected by feeding as small a dose as 2 gm. of glycine 1 hour before giving 3 gm. of benzoic acid. The theory can be tested in another way. If uric acid is actually accumulating in the blood after the administration

TABLE V

Effect of Glycine in Preventing the Decrease of Uric Acid Excretion following the Administration of Benzoic Acid

Time	Urine volume	Benzoic acid excreted as hippuric acid	Uric acid output per hr.	Remarks
	cc.	*gm.*	*mg.*	
6.00- 8.00 a.m.	46		15.3	2.0 gm. glycine taken at 8.00 a.m.
8.00- 9.00	40		15.4	3.0 gm. benzoic acid taken at 9.00 a.m.
9.00-10.00	40	0.80	18.8	
10.00-11.00	50	0.96	13.0	

of sodium benzoate, then a subsequent excess dose of glycine should cause a markedly increased elimination of uric acid. The results recorded in Table VI indicate the correctness of this assumption. Following the ingestion of 6 gm. of benzoic acid the hourly excretion of uric acid dropped to one-third of the normal. After the subsequent administration of a relatively small amount of glycine, 5.0 gm., a portion of which was moreover rendered inactive by conjugation with benzoic acid, a striking increase in the elimination of uric acid resulted.

These considerations may throw some light on the perplexing observations of Harding, Allin, Eagles, and Van Wyck (48) who found that diets which contain sufficient excess fat to produce ketosis increased the concentration of uric acid in the blood while

carbohydrates following such a diet lowered the uric acid of the blood and caused an increased elimination in the urine. It is quite possible that these findings may be explained by the theory of glycine deficiency. If this is true, it further suggests that glycine may be synthesized from a carbohydrate derivative, and that when a deficiency of available carbohydrates occurs as indicated by ketosis, the synthesis of glycine is diminished or entirely stopped. Probably glycuronic acid is another factor which must be considered. This problem will be investigated and reported later.

TABLE VI

Stimulating Effect of Glycine on Excretion of Uric Acid Following Its Suppression by Benzoic Acid

Time	Urine volume	Benzoic acid excreted as hippuric acid	Uric acid output per hr.	Remarks
	cc.	*gm.*	*mg.*	
7.00- 8.00 a.m.	54	0.02	22.5	
8.00- 9.00	69	0.67	12.5	3.0 gm. benzoic acid taken at 8.00 a.m.
9.00-10.00	52	0.75	6.9	3.0 gm. benzoic acid taken at 9.00 a.m.
10.00-11.00	160	1.34	10.0	5.0 gm. glycine taken at 10.00 a.m.
11.00-12.00	202	1.34	44.7	
12.00- 1.00 p.m.	51	0.41	20.0	

Excretion of Hippuric Acid

The second most important factor in the elimination of benzoic acid from the body is the capacity of the kidneys to excrete hippuric acid. Obviously, from the foregoing discussion it was seen that the hippuric acid elimination is faster than the synthesis of glycine required for the formation of hippuric acid. With an adequate quantity of glycine supplied in the diet, however, the excretion of hippuric acid reaches a maximum rate, which represents the ultimate power of the kidney to eliminate this product. Again the quantitative results indicate that the maximum rate of excretion depends on the size of the individual, that is, on the surface area. The urine volume does not appear to be of any

significance. As can be seen from Table II, the excretion of hippuric acid is essentially the same whether the compound is ingested directly, or whether benzoic acid with an adequate amount of glycine is fed. Glycine, furthermore, when administered with hippuric acid exerts no influence on its rate of excretion. This is contrary to Griffith's (49) observation on the rabbit, for in these animals glycine definitely did increase the rate of the elimination of ingested hippuric acid, but according to this author, hippuric acid is partially hydrolyzed in the intestines. Such a hydrolysis is rather unlikely in man since the absorption of hippuric acid is rapid, and it was furthermore found that a closely related substance, phenylaceturic acid, is excreted unchanged, whereas if it were hydrolyzed the phenylacetic acid should appear combined with glutamine (50).

The maximum output of benzoic acid as hippuric acid when the body has sufficient glycine varied in three individuals from 1.40 to 1.82 gm. per hour. Especially striking was the constancy of the maximum hourly output of hippuric acid in Subject Q in a series of experiments of rather widely varying conditions. Contrary to the findings of Lewis (21), Kingsbury and Swanson (37), and Bryan (29), the elimination of benzoic acid was rarely over 75 to 80 per cent complete in 6 hours. In agreement with Snapper (51) it was found that the last portion of the benzoic acid was eliminated slowly.

Conjugation of Benzoic Acid with Glycine

One factor which remains undetermined is the rate with which the conjugation of benzoic acid with glycine takes place. Unfortunately no method could be found by which the speed of this combination could be determined. When sufficient glycine is supplied with ingested benzoic acid, the elimination of hippuric acid is practically the same as when hippuric acid itself is taken by mouth. Thus, the union of glycine with benzoic acid must proceed at a faster rate than the excretion of hippuric acid. Since the rate of the excretion of hippuric acid in the fasting state depends primarily on the amount of glycine synthesized and secondarily on the capacity of the kidney to eliminate hippuric acid, the actual conjugation of benzoic acid with glycine becomes a factor of minor importance. The site of this synthesis, consequently, while still of theoretical interest loses in practical importance. While the evidence seems rather convincing that in the dog the synthesis of hippuric acid is confined solely to the kidney, it must be remembered that this animal is strikingly deficient in effecting this synthesis. Whether the synthetic power to combine glycine with benzoic acid is possessed by organs other than the kidney in man and those animals that produce much hippuric acid is a problem which requires further experimental work.

TABLE VII

Conjugation of Benzoic Acid in Fasting Showing Constant Rate of Hippuric Acid Excretion

Subject Q.

Time	Urine volume	Benzoic acid excreted as		
		Free	Hippuric acid	Glycuronic acid monobenzoate
	cc.	*gm.*	*gm.*	*gm.*
8.00–10.00 a.m.	30		0.04	0.05
10.00–12.00*	150	0.03	1.65	0.14
12.00– 2.00 p.m.	235	0.04	1.89	0.17
2.00– 4.00	140	0.03	1.93	0.28
4.00– 6.00	110	0.01	1.65	0.27
6.00– 8.00	110	0.01	1.55	0.27
8.00– 8.00 a.m.	275	0.08	3.10	0.65

* Took 10 gm. of benzoic acid at 10.00 a.m., and an additional 5.0 gm. at 11.00 a.m. Subject became nauseated in the afternoon, vomited once, and later developed a severe headache. Recovery was complete the following morning.

Conjugation of Benzoic Acid with Glycuronic Acid

The present study has thrown little light on the relationship of the conjugation of benzoic acid with glycine and with glycuronic acid. It was found that when a sufficiently large dose of benzoic acid was given so that the body's ability to synthesize glycine was strained, a portion of the benzoic acid (about 10 to 12 per cent) appeared in the urine combined with glycuronic acid. See Tables VII and VIII. It should be recalled that the writer has previously found that when a moderate dose of glycuronic acid monobenzoate is taken by mouth, none of the substance is excreted in the urine; the benzoic acid is eliminated as hippuric acid and the glycuronic

pathological changes in the liver. This can be illustrated by a patient who had a markedly enlarged liver and fairly typical signs of multiple neuritis, probably alcoholic in origin. When she received 5.0 gm. of benzoic acid by mouth, she excreted in 5.5 hours, 0.65 gm. of benzoic acid conjugated with glycuronic acid, and 2.5 gm. combined with glycine. Several months later when her clinical condition had shown great improvement, she no longer excreted glycuronic acid when the same quantity of benzoic acid was again given. Such a finding cannot be explained at present because of the lack of information concerning the physiological function of glycuronic acid.

The power of the kidney to eliminate hippuric acid may perhaps serve as a valuable test to determine the efficiency of this organ. Instead of determining the rate of excretion as Kingsbury and Swanson (54) have done, a more sensitive index may perhaps be found in the maximum hourly output of hippuric acid, since it appears to be fairly constant in normal individuals. This and related problems will be studied clinically and reported later.

SUMMARY

1. Quinic acid when ingested gives rise to a prolonged excretion of hippuric acid. Similar results are obtained after eating prunes, indicating that the precursor of benzoic acid in this and perhaps other fruits is quinic acid.

2. The most important factor in the excretion of hippuric acid following the ingestion of benzoic acid in the fasting state is the rate at which glycine is synthesized by the body. If the amount of benzoate taken is sufficiently large, the maximum rate of the synthesis of glycine can be determined, and it appears that it is a constant depending on the size of the individual.

3. The function of glycine in the body is discussed. The theory is presented that the decrease in uric acid excretion following the administration of sodium benzoate is due to the effect upon the kidney of a relative deficiency of glycine.

4. Glycuronic acid monobenzoate is excreted in small but definite amounts following moderately large doses of benzoic acid.

5. No method was found for determining the actual rate with which glycine is conjugated with benzoic acid.

6. The possible clinical application of studies on the conjugation of benzoic acid is briefly discussed.

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J. Biol. Chem. 98 (1): 157-169, 1932.

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND PHYSIOLOGICAL RESPONSE

III. FACTORS INFLUENCING THE EXCRETION OF URIC ACID*

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(Received for publication, July 18, 1932)

In a recent study, the writer (1) found that the decrease in the excretion of uric acid which results from the ingestion of sodium benzoate, can be completely prevented by supplying excess glycine. Since this amino acid, moreover, stimulates the excretion of uric acid, it was concluded that the action of benzoic acid depends on a depletion of the body's available glycine, thereby removing a stimulus necessary for the normal and efficient elimination of uric acid. Further studies made it apparent that this simple explanation did not suffice. New data have been obtained which indicate that there is a relationship between the conjugating mechanisms and uric acid excretion. With the presentation of these new findings, an attempt has been made to correlate as far as possible the various known factors which influence the excretion of uric acid.

The experimental procedure was simple. The neutralized drug dissolved in water was taken by mouth. A light breakfast consisting of coffee and a cruller was eaten 1 hour prior to the test, unless the nature of the experiment required fasting. A comparison of the uric acid excretion with and without breakfast showed practically no difference. The author served as the subject in most of the experiments, especially in those in which the work of other investigators was repeated. A number of the more crucial experiments, however, were also carried out on other normal adults, in order to obviate the danger of individual metabolic idiosyncrasies. Uric acid was determined by the Benedict-Hitchcock (2) method; the other analytical procedures were the same as employed in the previous papers of this series.

* This work was aided by a gift of Mrs. John L. Given in support of surgical research.

TABLE I

Factors Influencing Excretion of Uric Acid

Stimulating			Depressing		
Factor	Amount	Author	Factor	Amount	Author
	gm.			gm.	
Amino acids					
Glycine	10	Lewis, Dunn, and Doisy (3)	Lactic acid	12–14	Gibson and Doisy (4)
Alanine	12	" " " "			
Aspartic acid	18	" " " "	Glycolic acid	10	" " "
Glutamic "	20	" " " "			
Glucose	300	" and Corley (5)	Acetoacetic "	10–20	Quick
Glycerol	50	" " "	High fat diet (ketosis)		Hurdling, Allin, Eagles, and Van Wyck (6)
Pyruvic acid	16	Gibson and Doisy (4)	Fasting (ketosis)		Lennox (7)
High protein diet		Folin, Berglund, and Derick (8)	Benzoic acid	3–9	Lewis and Karr (9) Swanson (10)
Sodium chloride	30	" "	Phenylacetic acid	3	Quick
Salicylic acid		Extensive literature	Substituted benzoic acids	3	"
Cinchophen					
Neocinchophen					

All of the data were secured on hourly samples of urine. Previous workers as well as the writer have repeatedly observed that the rate of uric acid excretion is fairly constant during the first 4 to 5 hours of the morning. This period is therefore most suitable. The importance of short period collections cannot be emphasized too strongly. It is now fairly definitely established that the variations occurring in the uric acid excretion during a low purine régime are not due to any appreciable changes in production or destruction, but are dependent upon the excretory mechanism, which is apparently very sensitive to various stimuli. It can readily be seen that after a stimulatory factor has spent its activity, a compensatory decrease in the excretion may follow, and *vice versa*, when a depressor causing an accumulation of uric acid in the blood is removed, excess uric acid will be excreted. While these changes in the rate of excretion are readily demonstrated by short period urine collections, a 24 hour specimen may show nothing if the compensatory phase is completely within that period. Much of the older literature in which the data are based on 24 hour collections is therefore of negative value, and in some instances has even given rise to entirely erroneous conclusions.

DISCUSSION

The excretion of uric acid in normal subjects can be readily and markedly influenced by various factors. For the sake of conciseness, these are summarized in Table I.

On examining Table I, it immediately becomes apparent that a relationship exists between carbohydrate metabolism and uric acid excretion. The fact that Harding *et al.* (6) found a direct connection between the height of acetone excretion and the rise in blood uric acid suggests that either ketosis itself or the lack of an active antiketogenic carbohydrate derivative brings about a retention of uric acid. The increase in blood uric acid observed after a high fat diet, and in fasting, can thus be readily explained. It is probable that even the gradual decrease in uric acid output during short periods of fasting (Neuwirth (11)) can also be accounted for on this basis. The ingestion of acetoacetic acid also slightly decreases the excretion of uric acid. Acidosis is not a factor, since the ingestion of sodium acetoacetate actually increases the alkali reserve. Lennox (7) has, furthermore, found

that sodium bicarbonate does not relieve the retention of uric acid during fasting.

From a clinical point of view, it is interesting to note that Ricci (12) has recently found that blood uric acid in fasting diabetic patients was high in cases not treated with insulin, and normal in treated cases; and furthermore, that the elevation of blood uric acid following a high purine diet was more prolonged in diabetic than in normal subjects.

In considering the factors that cause an increase in uric acid elimination, one is inclined to theorize that an active antiketogenic carbohydrate derivative exerts a definite stimulatory action. Glucose, except in exceedingly large amounts, has no effect on the uric acid output. The action of glycerol is more pronounced; 50 gm. will cause a definite increase, but 10 gm. have no demonstrable effect. Pyruvic acid, in marked contrast, in amounts as small as 7 gm. will cause a relatively pronounced increase in uric acid acid excretion. The question naturally arises whether pyruvic acid itself is the active carbohydrate derivative responsible for the increased elimination of uric acid. While no definite answer can be given, it seems fairly certain that pyruvic acid must at least be closely related to this active derivative. Unfortunately, the action of dihydroxyacetone and of methylglyoxal was not determined. 30 units of insulin with a high carbohydrate intake had no immediate influence on the uric acid output.

Lactic acid, as well as glycolic acid, actually depresses the excretion of uric acid. The marked depression reported by Gibson and Doisy (4) was not obtained, however, in the present study. Significantly, lactic acid also depresses the production of glycuronic acid as measured by the excretion of glycuronic acid monobenzoate, whereas alanine and also insulin increase the production (unpublished results). These observations make it appear unlikely that lactic acid is a normal intermediary in the catabolism of glucose. Certainly, the difference in the actions of lactic acid and pyruvic acid on uric acid excretion make one doubt whether there is a ready interconversion of these two compounds. A study of the effect of strenuous exercise on uric acid excretion should, in view of the large production of lactic acid, yield valuable results.

The stimulatory effect of amino acids on uric acid excretion has been repeatedly observed; and it is reasonable to suppose that the

low blood uric acid recorded by Folin, Berglund, and Derick (8) in patients on a high protein and low purine diet is dependent upon the high amino acid intake. It should be noted that all of the amino acids thus far studied are glycogenetic. This leads one to wonder whether their action is really direct or whether it is through a carbohydrate derivative which they yield. In an earlier paper (13) the writer postulated that all glycogenetic amino acids probably yield a common derivative, which the organism can either utilize directly or can convert to glucose. On comparing the effects of approximately equivalent amounts of alanine and pyruvic acid, a striking similarity in their action on uric acid excretion can be seen. The same observation was made by Gibson and Doisy. Such results suggest that the stimulatory action may be due to the pyruvic acid derived from alanine rather than to the amino acid itself. If this assumption is correct, it must be concluded that alanine undergoes oxidative deaminization.

While the action of glycine on uric acid elimination is not specific, it is, nevertheless, more effective than other amino acids. In the first place, glycine, like other glycogenetic amino acids, stimulates the output of uric acid; and in the second place, it prevents and counteracts the depressing action of benzoic acid and substituted benzoic acids on the excretion of uric acid.

The striking action of aromatic acids on the elimination of uric acid has received comparatively little attention. Lewis and Karr (9) were the first to observe that benzoic acid caused a marked drop in the excretion of uric acid. Their work was corroborated by Swanson (10), who found a rise in the blood uric acid concomitant with the decrease in excretion. He disagreed with the conclusion of Lewis and Karr that the decreased excretion is brought about by a conversion of uric acid to glycine, but concluded that the excretion of uric acid is probably slightly retarded whenever large amounts of hippuric acid are being eliminated. Swanson, furthermore, found that benzoic acid did not cause a retention of urea, and Lewis and Karr noted a normal excretion of creatinine. While these findings indicate that benzoic acid exerts a direct action on the excretion of uric acid rather than a general nephrotoxic effect resulting in a generalized nitrogen retention, it must be remembered that uric acid is eliminated with greater difficulty than the other nitrogenous constituents and in early nephritis may be the only one that is retained.

In the present study it was found that not only benzoic acid, but substituted benzoic acids and phenylacetic acid as well, produce a prompt, marked, and prolonged decrease in the excretion of uric acid, and furthermore, that the period of depression in general coincides with the time required by the organism to eliminate the particular drug. The action of benzoic acid is of relatively short duration, while that of *o*-toluic acid and other ortho-substituted benzoic acids, which the body eliminates with difficulty, is much more pronounced. It will be recalled that in a previous paper of this series (14) it was found that substitution of benzoic acid in the ortho position greatly inhibits the conjugation with glycine. Phenylacetic acid, which is also excreted much more slowly than benzoic acid, likewise exerts a more prolonged depressing action on uric acid excretion. While the effect of 3 gm. of benzoic acid lasts only 2 hours, that of a similar amount of phenylacetic acid exceeds 5 hours.

It is significant that the depressing action of these aromatic acids completely abolishes the stimulatory effect of glycerol, pyruvic acid, and the various amino acids, with one exception. A small excess of glycine will counteract the action of benzoic acid completely, and that of *o*-toluic acid partially, but it is ineffective against phenylacetic acid (Tables II and III). It will be recalled that phenylacetic acid in the human is not conjugated with glycine but with glutamine.

While the stimulatory action of both pyruvic acid and alanine is entirely abolished by phenylacetic acid, it should be noted that the period of uric acid retention is shortened by either compound to approximately one-half. Preliminary studies on the rate of excretion of conjugated phenylacetic acid, moreover, show a definite increase when either alanine or pyruvic acid is fed. This leads one to conclude that pyruvic acid can furnish the precursor for glutamine. It is rather interesting that glutamic acid is ineffective.

While the foregoing results show a definite relationship between uric acid excretion and the conjugative mechanisms, it is not possible at present to offer any explanation. A further study of the action of other aromatic acids is desirable, especially from a clinical point of view. Such conditions as gout and eclampsia, in which there is a specific retention of uric acid, may perhaps have as their etiological factor a toxin similar to these common aromatic

TABLE II

Various Factors Influencing Excretion of Uric Acid

Uric acid excretion						Subject	Summary of experiment
1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.		
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*		
23.0	23.2	22.1	20.5	18.6		A	Fasting
23.0	27.0	24.3	23.0			"	"
24.0	23.0	21.3	25.0			"	Light breakfast
31.0	26.0	28.1	25.2			B	" "
25.0	20.5	21.0	30.7			A	20 gm. acetoacetic acid. Fasting
30.0	26.6*	20.0				C	10 " " " "
21.0	18.4*	18.6	17.6			D	10 " " " "
29.0	26.7*	25.0	25.2			A	30 units of insulin. 100 gm. sucrose
20.1	17.4	17.6	20.0			"	10 gm. lactic acid
20.0	20.4	20.5	17.4			"	10 " " " Fasting
33.4	21.8*	21.6	21.0			B	15 " " " "
9.1	6.2	20.2	22.0			A	3 gm. benzoic acid
22.0	8.6	11.7	12.4	12.0	15.3	"	3 " phenylacetic acid
18.5	9.2	11.0	12.3			"	3 " " " and 3 gm. glycine
30.0	27.0	20.5	18.8			"	50 gm. glycerol
25.8	8.0	15.6				"	50 " " and 3 gm. benzoic acid
31.6	35.0	36.0	40.0			"	7 gm. pyruvic acid
12.0	7.2	10.9	21.2			"	7 " " " and 3 gm. phenylacetic acid
29.6	38.6	28.0	22.2			"	10 gm. alanine
14.3	7.7	10.7	22.0			"	10 " " and 3 gm. phenylacetic acid
22.5	28.0	20.4				"	8 gm. glutamic acid
24.2	11.5	14.5	20.2			"	8 " " " and 3 gm. benzoic acid
17.6	11.2	10.9	11.3			"	8 gm. glutamic acid and 3 gm. phenylacetic acid
25.8	27.2	27.2	24.0			"	7 gm. aspartic acid
23.0	11.6	24.0				"	7 " " " and 3 gm. benzoic acid

* Drug taken at the beginning of the 2nd hour.

acids, or a damaged conjugative mechanism as a result of which the organism is deprived of its ability to handle the simple toxins arising from intestinal putrefaction and other common sources.

No attempt was made to investigate the effect of sodium chloride on uric acid excretion. Folin, Berglund, and Derick (8) stated that 30 gm. increased the output, but they gave no actual data. Harding, Allin, and Van Wyck (15) found that 15 gm. decreased the level of blood uric acid. This they attributed to an increased hydration of the blood. Further work, however, is necessary before one can arrive at any definite conclusion.

In view of the characteristic action of salicylic acid on uric acid excretion, it seemed desirable to make a comparative study of the three hydroxybenzoic acids, the conjugation of which has been

TABLE III

Conjugation of Ortho-Substituted Benzoic Acids and Their Effect on Output of Uric Acid

Time	*o*-Toluic acid				*o*-Chlorobenzoic acid 2.0 gm.			*o*-Methoxybenzoic acid 3.7 gm.		
	0.5 gm.	1.0 gm.	3.3 gm.*	3.3 gm. and 5 gm. glycine	Uric acid	Excretion		Uric acid	Excretion	
						Free	With glycine		Free†	With glycine
	Uric acid excretion									
hrs.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*	*gm.*	*mg.*	*gm.*	*gm.*
1	24.0	26.0	10.8	19.8	12.0	0.20	0.13	15.8	0.05	0.05
2	23.0	15.7	8.3	17.8	11.0	0.24	0.27	14.1	0.06	0.06
3	21.0	14.5	8.5	14.0	17.0	0.22	0.18	9.5	0.02	0.06
4	25.0	17.0	13.6	15.5	19.0	0.13		25.0‡	0.06	0.21

* The average excretion of *o*-toluic acid per hour was: free 0.01 gm., combined with glycuronic acid 0.21 gm., and combined with glycine 0.08 gm.

† Also combined with glycuronic acid.

‡ 3 gm. of glycine were taken at the end of the 3rd hour.

reported in the preceding paper of this series (16). Hanzlik's (17) excellent review of the literature on salicylic acid makes it unnecessary to discuss the individual papers. Since nearly all of the workers drew their conclusions from analytical results obtained on 24 hour urine specimens, it was felt that a study made on the hourly excretion should disclose the transient and finer variations which hitherto have been overlooked.

A comparison of the action of the three hydroxybenzoic acids on the output of uric acid discloses striking differences (Table IV). Salicylic acid promptly and markedly increases the excretion of

uric acid. A similar but much less marked effect is produced by *p*-hydroxybenzoic acid, whereas *m*-hydroxybenzoic acid, like benzoic acid, distinctly depresses the excretion. It should be mentioned that Denis (18) found that *p*-hydroxybenzoic acid did not affect the excretion of uric acid, and Rockwood (19) likewise concluded that neither the para nor the meta compound had any effect, although his data suggested a slight suppression by the latter acid. Both studies were carried out on 24 hour collections of urine. In attempting to correlate the action of these acids with

TABLE IV

Effect of Hydroxy- and Methoxybenzoic Acids on Excretion of Uric Acid*

Time	*p*-Hydroxybenzoic acid				*m*-Hydroxybenzoic acid				*o*-Hydroxybenzoic acid			
	Urine volume	Excretion of			Urine volume	Excretion of			Urine volume	Excretion of		
		Uric acid	Sodium chloride			Uric acid	Sodium chloride			Uric acid	Sodium chloride	
hrs.	*cc.*	*mg.*	*gm.*	*per cent*	*cc.*	*mg.*	*gm.*	*per cent*	*cc.*	*mg.*	*gm.*	*per cent*
1	33	30.0	0.32	0.96	30	11.1	0.32	1.12	22	35.0	0.22	1.00
2	57	32.0	0.55	0.96	45	7.5	0.47	1.04	35	58.0	0.34	0.96
3	37	21.8	0.46	1.24	35	9.7	0.42	1.20	49	46.0	0.49	1.00
4	33	22.5	0.51	1.56	40	16.3	0.54	1.36	55	44.0	0.59	1.08
	p-Methoxybenzoic acid				*m*-Methoxybenzoic acid				*o*-Methoxybenzoic acid			
1	33	23.0			34	12.6			26	16.0		
2	43	22.6			46	9.5			31	14.0		
3	35	20.0			51	10.4			18	9.5		
4	28	20.0			55	22.0			43	25.2†		

* 3.5 gm. of the hydroxybenzoic acid, and 3.7 gm. of the methoxybenzoic acid were taken.

† 3 gm. of glycine were taken at the end of the 3rd hour.

their conjugation, it will be recalled that salicylic acid is excreted very slowly and entirely uncombined, *p*-hydroxybenzoic acid is conjugated partly with glycine and partly excreted uncombined, while *m*-hydroxybenzoic acid is combined mainly with glycine. The pharmacological action appears to depend on the presence of a free carboxy and hydroxy group in the molecule. One must bear in mind, however, that the metabolism of the glycuronic acid associated with these groups may be one of the important underlying factors upon which the activity of these compounds depends.

By converting the hydroxy radical to the inactive methoxy group, the stimulatory action is abolished. In the case of the ortho compound a marked depressing action on uric acid excretion is actually produced.

TABLE V

Action of Salicylic Acid on Uric Acid Output

Salicylic acid taken.....	0.5 gm.	1.0 gm.	1 gm., 3 gm. benzoic acid
Time	Uric acid excretion		
hrs.	*mg.*	*mg.*	*mg.*
1	21.4	27.4	21.4
2	23.2	43.0	28.0
3	40.0*	37.0	18.5
4	58.0	37.0	36.0

* 1.5 gm. of additional salicylic acid were taken at the end of the 2nd hour.

TABLE VI

Action of Cinchophen and Neocinchophen on Output of Uric Acid

Time	Cinchophen, 1.0 gm.			Neocinchophen, 1.2 gm.		
	Urine volume	α_D^*	Uric acid	Urine volume	α_D^*	Uric acid
hrs.	*cc.*	*degrees*	*mg.*	*cc.*	*degrees*	*mg.*
1	24	−0.20	30.0	21	−0.15	30.0
2	28	−0.30	53.6	25	−0.20	41.6
3	35	−0.30	59.6	23	−0.20	38.4
4	36	−0.35	61.5	22	−0.15	38.4
5	39	−0.35	44.5	22	−0.20	53.5
6	51	−0.30	41.8	29	−0.15	47.5
7	30	−0.40	28.6	34	−0.20	39.0
8	44	−0.32	32.6	16		33.6

* The rotation was observed in a 1 dm. tube. A slight levorotation, such as was observed after taking neocinchophen, is found in normal urine.

The amount of salicylic acid necessary to produce its effect is surprisingly small. For a man weighing 50 kilos, 1 gm. caused nearly the maximum effect, while a 0.5 gm. dose produced no stimulation of uric acid excretion whatsoever (Table V). It is interesting to note that salicylic acid and benzoic acid mutually counteract the effects of each other

The action of cinchophen (phenylcinchonic acid) and neocinchophen (ethyl ester of *p*-methylcinchonic acid) appears to be similar to that of salicylic acid as seen from the results recorded in Table VI. The structural similarity between salicylic acid and cinchophen has been pointed out in a previous paper (13). It will be recalled that the second aromatic ring, condensed as in naphthalene, produces the same effect on the α-carboxyl group as ortho substitution. Consequently, conjugation with glycine is inhibited, and this probably explains the slow elimination of cinchophen. There is evidence that both cinchophen and neocinchophen are readily oxidized to a hydroxy compound. Dohrn (20), and Rotter (21) have isolated a hydroxycinchophen from the urine, and Fürth and Kuh (22) have obtained a dihydroxyneocinchophen. It is probable that neither cinchophen nor neocinchophen becomes therapeutically active until it is oxidized to the hydroxy compound. This view has also been expressed by Rotter. It should be noted that after the ingestion of cinchophen, the urine becomes definitely levorotatory. The constancy of this rotation suggests that a levorotatory compound is eliminated at a slow and fixed rate. When cinchophen is fed to a dog, a very marked levorotation of the urine is found. While the nature of the compound has not been determined, one is inclined to consider it a conjugation product, since it is difficult to see how any change in the cinchophen molecule can give rise to an asymmetrical carbon atom. There is no evidence of a levorotatory compound being produced after feeding neocinchophen.

No definite explanation for the action of salicylic acid, cinchophen, and neocinchophen can be given, but it seems quite probable that the action of these three drugs is essentially the same. From the results in Tables IV and VI, it can be seen that the excretion of uric acid is entirely independent of the urine volume, in sharp contrast to the output of chlorides, which is almost directly dependent upon the volume. Whether the extremely small volume of urine occurring after the ingestion of neocinchophen is due to the drug, cannot be answered until further studies have been made.

SUMMARY

1. The relationship between carbohydrate metabolism and uric acid output is discussed, and the possibility that an active carbo-

hydrate derivative may be necessary for the normal excretion of uric acid is considered.

2. Benzoic acid, various substituted benzoic acids, and phenylacetic acid depress the excretion of uric acid, and the duration of their action coincides with the time required for their conjugation and elimination.

3. The stimulating action on uric acid elimination of glycerol, pyruvic acid, and the various amino acids is completely inhibited by these aromatic acids; nevertheless, both pyruvic acid and alanine greatly shorten the period of effectiveness of phenylacetic acid. The depressing action of benzoic acid and the various substituted benzoic acids is prevented, at least partially, by excess glycine.

4. The excretion of uric acid is markedly increased by salicylic acid, moderately by *p*-hydroxybenzoic acid, and depressed by *m*-hydroxybenzoic acid.

5. The replacement of the hydroxy group by a methoxy radical completely abolishes the stimulatory action of the *p*- and *o*-hydroxybenzoic acids, indicating that the pharmacological action of these compounds is dependent upon a free hydroxy and carboxy group in the molecule.

6. The action of cinchophen and neocinchophen on the excretion of uric acid appears to be similar to that of salicylic acid. After the ingestion of cinchophen, a levorotatory substance appears in the urine.

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6284

Conjugation of Benzoic Acid with Glycine, a Test of Liver Function.

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In a recent study on the conjugation of benzoic acid in man, the author found that the excretion of hippuric acid proceeded at a constant rate irrespective of the amount of sodium benzoate administered.[1] Since it was further found that the output of hippuric acid was greatly increased by feeding glycine or foods rich in glycine such as gelatine, it became obvious that the rate of hippuric acid formation is dependent upon the speed with which the body can synthesize glycine. It was determined that a normal adult can produce enough glycine to conjugate from 0.90 to 1.30 gm. of benzoic acid per hour.

Since the main site of the synthesis of glycine is presumably the liver, it was considered probable that certain types of liver damage might show a diminution in the hourly excretion of hippuric acid. On this basis a test was carried out as follows: 5.9 gm. of sodium benzoate dissolved in 150 cc. of water is administered 1 hour after a breakfast consisting of coffee and toast. Complete hourly specimens of urine are collected for 4 hours. Hippuric acid is determined by the author's method.[2] A simpler method which is sufficiently accurate for clinical purposes can be employed. In this procedure the hourly specimen is measured, transferred to a small beaker, and acidified with concentrated HCl until acid to Congo red. (1 cc. of the acid is usually sufficient.) The solution is vigorously stirred until the hippuric acid has completely crystallized out, and then is allowed to stand at room temperature for 1 hour. The hippuric acid is filtered off on a Buchner funnel or a small filter plate, washed with cold water, allowed to air dry, and finally weighed. (Weighing to the second decimal place is sufficiently accurate.) To obtain the total hippuric acid, one adds to the weight of the precipitated crystals, the calculated amount remaining in solution. One hundred cc. of urine will hold in solution approximately 0.33 gm. of hippuric acid. If the volume of any specimen exceeds 150 cc., it should be slightly acidified with acetic acid, and concentrated on a water bath to about 50 cc. before precipitating the hippuric acid.

In applying the test to various liver diseases, the most striking

[1] Quick, A. J., *J. Biol. Chem.*, 1931, 92, 65.

[2] Quick, A. J., *J. Biol. Chem.*, 1926, 67, 477.

results were obtained in cases of toxic jaundice including catarrhal jaundice, and in obstructive jaundice of moderately long standing. In all these cases a definite diminution in the rate of hippuric acid excretion occurred, and significantly, the hourly output often remained low long after the jaundice had cleared up. In Table I a few typical results are recorded.

TABLE I.
Hourly Excretion of Hippuric Acid.

Time hr.	Normal I gm.	Normal II gm.	Normal III gm.	Obstructive Jaundice gm.	Obstructive Jaundice gm.	Catarrhal Jaundice gm.	Catarrhal Jaundice gm.
1	1.29	0.43	0.81	0.54	0.43*	0.23	0.35*
2	1.96	1.96	1.46	0.93	0.56	0.51	0.51
3	0.82	1.28	1.51	0.93	0.54	0.65	0.44
4	0.28	0.79	1.49	0.81	0.70	0.54	0.69

* 10 days after the first test.

J. Clin. Invest. 36: 297-302, 1957.

THE CHEMICAL ESTIMATION OF ACYL GLUCURONIDES AND ITS APPLICATION TO STUDIES ON THE METABOLISM OF BENZOATE AND SALICYLATE IN MAN

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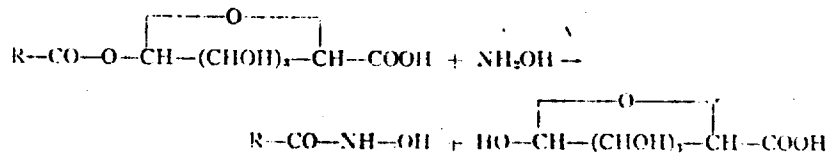
(Submitted for publication September 17, 1956; accepted October 18, 1956)

Structurally, glucuronides are the condensation products of hydroxyl-bearing compounds with the first, hemiacetal carbon of D-glucuronic acid. Two groups have been described (1). Alcohols and phenols form *ethereal* glucuronides, which are resistant to hydrolysis by mild alkali and do not reduce alkaline copper reagents. Carboxylic acids form *ester* glucuronides, which are easily split by mild alkali to liberate free glucuronic acid, a reducing agent. The term *acyl glucuronide* is introduced here for members of the latter group to denote clearly conjugation via the carboxyl group.

Another chemical reaction characteristic of the acyl glucuronides is described in this report. At room temperature and neutral pH, the acyl group can be transferred to hydroxylamine to form characteristic hydroxamic acids (Reaction I).



On addition of acid ferric chloride solution, hydroxamic acids yield colored products which can be measured spectrophotometrically (2). Thus, conversion to the stable hydroxamates provides a sensitive, chemical method for estimating the relatively unstable acyl glucuronides. The derivatives are easily extracted into organic solvents, and can be identified by chromatographic and other techniques. Non-acyl glucuronides do not form hydroxamic acids.

The methods reported here have permitted more extensive studies on the metabolic fate of aromatic acids in man. Since urinary glucuronides have been found following the ingestion of benzoate, salicylate, and probenecid (1, 3), observations were made on the metabolism of these clinically important drugs.

METHODS AND MATERIALS

Drug experiments. The subject was a healthy 28-year-old white male, weighing 60 kilos. His diet was not rigidly controlled, but remained fairly uniform throughout the period of investigation; all experiments were begun in the fasting state. At least 2 weeks elapsed between successive doses of drug. On the morning of an experiment, a control urine was collected over a period of several hours, and the appropriate drug was ingested as a solution of its sodium salt. Subsequently, timed voided urine samples were tested immediately for acyl glucuronides and stored at 5° C until completion of the remaining estimations. When necessary, the excretory rates of urinary drug metabolites were corrected for pre-ingestion, endogenous rates.

Estimation of acyl glucuronides. These compounds are estimated by conversion to their hydroxamates, using modifications of the procedure of Lipmann and Tuttle (2). The pH of the neutralized hydroxylamine reagent is critical and should be 7.0 ± 0.2. Stock solutions of NaOH (14 per cent) and hydroxylamine hydrochloride (28 per cent) are titrated against each other with a Beckman glass electrode pH meter to determine the exact proportions required. Urine samples containing 0.5 to 2.0 μmoles of acyl glucuronide are incubated with 0.5 ml. of freshly prepared neutral hydroxylamine for 2 hours at room temperature before color development and comparison with authentic hydroxamate standards. Figure 1 demonstrates the complete conversion of benzoyl glucuronide to benzoyl hydroxamate under these conditions.

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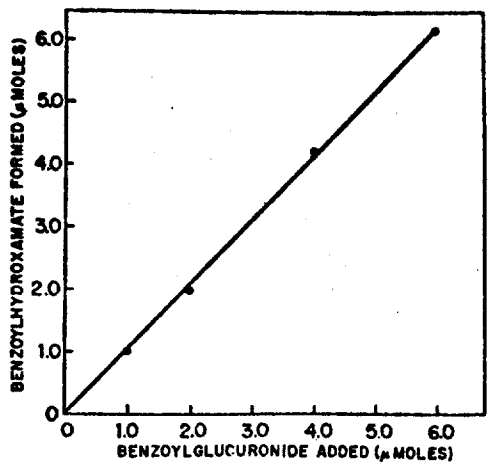

FIG. 1. QUANTITATIVE CONVERSION OF BENZOYL GLUCURONIDE TO BENZOYL HYDROXAMATE

The indicated amounts of crystalline benzoyl glucuronide in 0.5 ml. water were mixed with 0.5 ml. neutral hydroxylamine and incubated 2 hours at room temperature. The volume was adjusted to 2.0 ml. with water, and 0.5 ml. of 3 N HCl followed by 0.5 ml. of 5 per cent $FeCl_3 \cdot 6H_2O$ in 0.1 N HCl added. The optical density at 540 mμ was compared with that of a benzoyl hydroxamate standard.

To estimate salicyl acyl glucuronide (SAG) in urine by this procedure, 6 N HCl is substituted for 3 N HCl to minimize the formation of purple products arising from salicylate and salicylurate. Further, a correction is made in each estimation by subtracting the optical density at 540 mμ of a similarly treated, hydroxylamine-free control.[2]

The specificity of hydroxamate formation was studied with a series of 9 glucuronides, using 2.0 μmoles of each in the assay. (The conditions are described in the legend to Figure 1). Only benzoyl- and o-methoxybenzoyl glucuronides yielded such products, and no reaction was observed with the glucuronides of salicylamide, N-acetyl salicylohydrazine, 3-hydroxycoumarin, pregnandiol, phenolphthalein, menthol, and borneol.

Identification of urinary acyl glucuronides. Urinary hydroxamate-forming materials appear after the administration of benzoate and salicylate. The following evidence identifies these as benzoyl- and salicyl acyl glucuronides (SAG), respectively. These compounds are completely hydrolyzed by 0.1 N NaOH in 10 minutes at room temperature, and 84 per cent hydrolyzed by incubation with bacterial β-glucuronidase (250 units per ml.) for 60 minutes at 38° C. A single hydroxamate spot was detected for each compound by paper chromatography of urinary aliquots, using Whatman No. 1 paper and the ascending technique, with water-saturated n-butanol: glacial acetic acid (80:20, v/v) as the solvent system. These spots reduce aniline phthalate (4), and give a positive carbazole reaction for hexuronic acid (5) after elution into water. The benzoate metabolite moves at a rate (Rf 0.67) similar to that of crystalline benzoyl glucuronide. The salicylate conjugate (Rf 0.72) liberates free salicylate on hydrolysis in 6 N HCl at 100° C for 1 hour.

The hydroxamate derivatives of these urinary metabolites were extracted at neutral pH into ether and chromatographed on paper as described above, with water-saturated n-butanol as the developing solvent. Movement of the benzoyl derivative (Rf 0.79) corresponded to that of an authentic sample of benzoyl hydroxamate, and that of the salicyl compound (Rf 0.85) to crystalline salicyl hydroxamate.

That the urinary SAG is a monoglucuronide has been established in the following manner: its hydroxamate is completely extractable into ether, while that of the diglucuronide would be ether-insoluble. In addition, the hydroxamate is chromatographically homogeneous and identical in behavior with synthetic salicyl hydroxamate.

Estimation of urinary salicyl metabolites. In addition to SAG, salicyl phenolic glucuronide (SPG), salicylurate, and total salicyl were estimated.

SPG accounts for the difference between the quantity of SAG and the total salicyl glucuronide excreted. The latter is estimated by the salicylate liberated by bacterial β-glucuronidase. An aliquot of urine, adjusted to pH 2, is extracted twice with an equal volume of ether to remove salicylate and salicylurate, and readjusted to pH 6.0. Samples are withheld for initial salicylate (6) and SAG determinations. Five hundred units of β-glucuronidase are added to each ml. of extracted urine, the mixture is incubated at 38° C for 8 hours, an identical amount of enzyme again added and the incubation repeated.[3] Finally, 10 per cent perchloric acid filtrates are prepared and the free salicylate concentrations again determined.

$$\text{SPG} = \text{final salicylate} - \text{initial salicylate} - \text{SAG}$$

Salicylurate is estimated by a paper chromatographic technique. Suitable volumes of urine are quantitatively applied in duplicate to Whatman No. 1 paper for ascend-

[2] A satisfactory method of eliminating blank values due to salicylate and salicylurate is to extract the reaction mixture after formation of salicyl hydroxamate with 4 volumes of ether at neutral pH. Salicylate and salicylurate remain behind, while over 90 per cent of the hydroxamate is extracted. An aliquot of the ether is evaporated and color developed with HCl and ferric chloride. Values for acyl glucuronide in salicyl urine were identical, when estimated by both this procedure and that described in the text.

[3] Enzyme-treated urine was further hydrolyzed in 6 N HCl at 100° C for 3 hours, and free salicylate again estimated. The results indicated that enzymatic hydrolysis was at least 80 per cent complete under the conditions described in the text. Enzymatic hydrolysis is preferable to acid hydrolysis since specificity for glucuronides is obtained.

ing chromatography, with n-butanol: ethanol (40:11, v/v) saturated with an ammonium carbonate buffer (7) as the solvent system. The dried paper chromatograms are viewed with an ultra-violet lamp, and the fluorescent salicylurate areas (Rf 0.31) outlined and eluted into water. (The salicylate fluorescent area corresponds to Rf 0.66.) To 2.8 ml. of eluate, containing about 0.5 μmole salicylurate, 0.2 ml. of 0.1 M ferric chloride in 0.07 N HCl is added. The optical density of the purple products is measured at 540 mμ in the Beckman model B spectrophotometer. Recoveries of known quantities of salicylurate, chromatographed simultaneously, have ranged from 90.0 to 102.0 per cent.

The procedure of Lester, Lolli, and Greenberg (6) was followed in estimating total urinary salicyl.[4]

Corrected total salicyl = observed total salicyl + salicylurate × 0.676 × 0.225 + salicylurate × 0.676 × 0.775 − salicylurate × 0.676 × 0.775 × 0.84

Corrected total salicyl = observed total salicyl + salicylurate × 0.236. The salicylurate concentration is estimated by the chromatographic method described in the text.

Other methods. Chemical estimations were employed for hippurate (9), hexuronic acid (5), and probenecid (3). Probenecid conjugates were hydrolyzed by refluxing in 4.0 N H_2SO_4 for 60 minutes. Optical density measurements were made in the Beckman model B spectrophotometer.

Materials. The following compounds were prepared according to published methods and recrystallized from hot water: benzoyl glucuronide (10), m.p. 181–2° (decomp.) (Pryde and Williams: 183° [11]); salicyluric acid (12), m.p. 166° (Quick: 167° [12]); salicyl hydroxamic acid (13), m.p. 168° (Jeanrenaud: 168° [13]). A reference standard solution of benzoyl hydroxamate was prepared by reaction of neutral hydroxylamine with recrystallized benzoic anhydride (14). Crystalline salicylamide glucuronide (174°), o-methoxybenzoyl glucuronide (123–4°), and N-acetyl salicylohydrazine glucuronide (208–10°) were gifts from Doctor R. T. Williams to Captain R. M. Dowben; 3-hydroxycoumarin glucuronide (207–8°) was a gift from Captain R. M. Dowben; menthol-, borneol-, pregnandiol-, and phenolphthalein glucuronides were purchased from the Sigma Chemical Company, as was the bacterial β-glucuronidase.

[4] In this procedure aliquots of salicyl urine are hydrolyzed to liberate salicylic acid, which is extracted into ether and estimated by reaction with ferric chloride. The "total salicyl" concentration so measured requires complete hydrolysis of all salicyl conjugates. However, 67.6 per cent of the glycine conjugate remains unhydrolyzed, as determined by the liberation of glycine (8) in similarly treated salicyluric acid solutions. Hence it is necessary to correct the observed total salicyl concentration for: 1) incomplete ether extraction of salicylurate (77.5 per cent as compared to 100 per cent for salicylate), and 2) relative intensity of the colored products formed with ferric chloride (84.0 per cent for salicylurate as compared to salicylate).

RESULTS

Ingestion of benzoate

In individual experiments, the subject was fed 6.9, 13.9, 34.7, and 69.3 millimoles of sodium benzoate.[5] Complete elimination of the drug as urinary hippurate and benzoyl glucuronide ensued in each instance, with final recoveries of 98 to 104 per cent. The relative amounts of benzoate conjugated with glycine and with glucuronic acid varied with the dosage, as shown in Figure 2. The glucuronide moiety increased progressively from 0.4 per cent (0.03 millimoles) to 3.0 per cent (2.08 millimoles). Hippurate accounted for the bulk of conjugated benzoate throughout this dose range.

The cumulative urinary recovery of each metabolite is plotted in Figure 3 for a representative experiment. The recoveries of acyl glucuronide and hippurate ran a parallel course, a characteristic observed at all the dosage levels examined. Complete excretion of benzoate required 3 to 4 hours after the two smaller doses, and 10 to 14 hours after the larger amounts.

As indicated in Table I, the maximal urinary excretory rate (μmoles per minute) achieved in a given experiment by either metabolite depended on the dose of benzoate. Limiting values for hip-

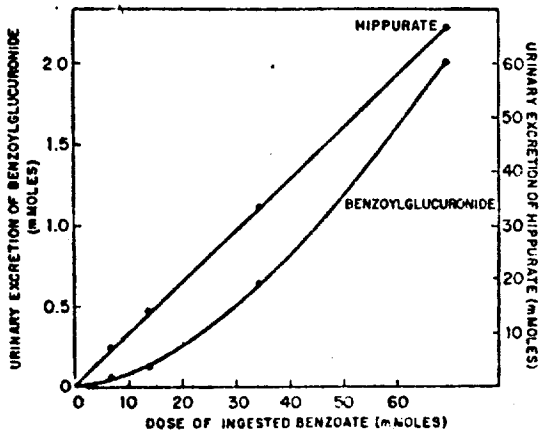

FIG. 2. QUANTITIES OF URINARY HIPPURATE AND BENZOYL GLUCURONIDE RECOVERED AFTER VARIOUS DOSES OF BENZOATE

The right- and left-hand vertical scales refer to hippurate and benzoyl glucuronide, respectively.

[5] Corresponding to 1.0, 2.0, 5.0, and 10.0 grams.

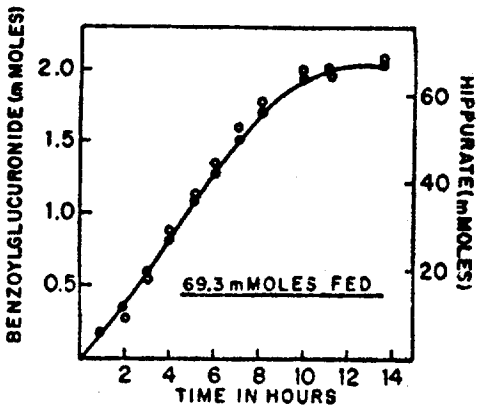

FIG. 3. CUMULATIVE URINARY RECOVERIES OF HIPPURATE (—●—●—) AND BENZOYL GLUCURONIDE (○)

purate excretion were approached at a dose of 13.9 millimoles. Quick (15) has demonstrated that limitations in the availability of glycine may account for this, and the peak rate attained in these experiments (130.5 μmoles per minute) is within his range for maximal rates of glycine mobilization in man (122 to 155 μmoles per minute). By contrast, the maximal excretory rate of benzoyl glucuronide was approximately proportional to the benzoate dose throughout the range studied. It is significant that the glucuronide was detected at a dosage level (6.9 millimoles) far below that required for maximal hippurate excretion. These observations fail to support the supposition (15) that the glucuronide synthesis is a reserve detoxication mechanism, operating only when the glycine supply is exceeded. Further pertinent evidence is obtained from the data in Figure 3. After the seventh hour in this experiment, the excretion of hippurate was well below its maximal rate, yet significant benzoyl glucuronide excretion persisted.

TABLE I

Maximal rates of hippurate and benzoyl glucuronide excretion following various doses of benzoate

Dose of sodium benzoate		Maximal urinary excretory rate (μmoles per min.)	
(grams)	(millimoles)	Hippurate	Benzoyl glucuronide
1.0	6.9	54.8	0.4
2.0	13.9	108.2	1.0
5.0	34.7	111.3	2.6
10.0	69.3	130.5	5.5

Ingestion of salicylate

Four experiments were performed, the subject ingesting 7.5, 15.0, 22.5, and 30.0 millimoles of sodium salicylate.[6] The relative quantities of urinary salicyl metabolites excreted after these doses are listed in Table II. With doses of 15.0 to 30.0 millimoles about 50 per cent of the amount fed was recovered as urinary salicyl in 24 hours (and 85 per cent at the end of 48 hours). Salicylurate made up 60 per cent of this, and the glucuronide, 30 per cent, values which agree closely with previous observations (12, 16). SAG and SPG were present in about equal amounts. Cumulative urinary recoveries of the salicyl metabolites, in a representative experiment, are depicted in Figure 4. Whereas total salicyl, salicylurate, and SAG ran a course parallel to each other with time, the recovery of SPG was relatively lower in the early hours and higher thereafter. The maximal rates of excretion (μmoles per minute) of the urinary products varied with the doses, as shown in Table II; limiting values for these rates were not achieved in this range.

Characteristic differences in the excretory patterns of SAG and SPG were observed in all the

[6] Corresponding to 1.0, 2.1, 3.1, and 4.2 grams

TABLE II

Urinary metabolites of salicylate following various oral doses

Dose millimoles	Urinary salicyl recovered in 24 hours, per cent of dose	Urinary metabolites recovered in 24 hours, per cent of total urinary salicyl			Maximal rates of urinary excretion (μmoles per min.)			
		Salicylurate	SAG	SPG	Total salicyl	Salicylurate	SAG	SPG
7.5	78.6	64.5	13.1	12.7	4.5	3.0	0.8	0.6
15.0	54.5	62.8	14.8	21.0	5.6	4.1	1.0	1.4
22.5	49.4	74.0	14.3	15.1	8.0	6.0	1.5	1.5
30.0	50.8	59.6	16.4	12.1	11.1	6.8	1.9	1.7

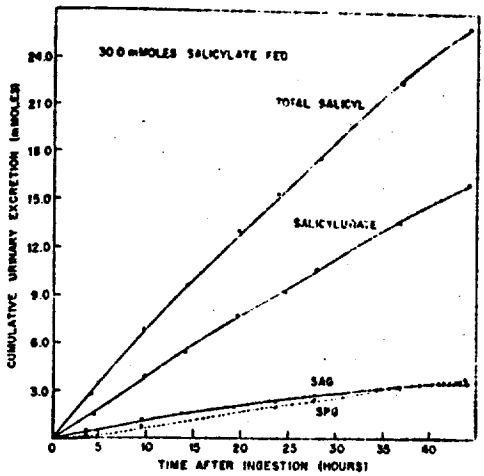

FIG. 4. CUMULATIVE URINARY RECOVERIES OF SALICYL METABOLITES

experiments. These are illustrated in Figure 5. The peak SAG excretory rates were observed within 5 to 10 hours after ingestion, with a rapid fall thereafter. By contrast, the SPG peak was obtained after 20 to 30 hours, and the subsequent fall-off was more gradual.

Ingestion of probenecid

Seven and one-half millimoles (2.1 grams) of this drug (sodium p-(dipropylsulfamyl) benzoate) were administered to the subject. At the end of

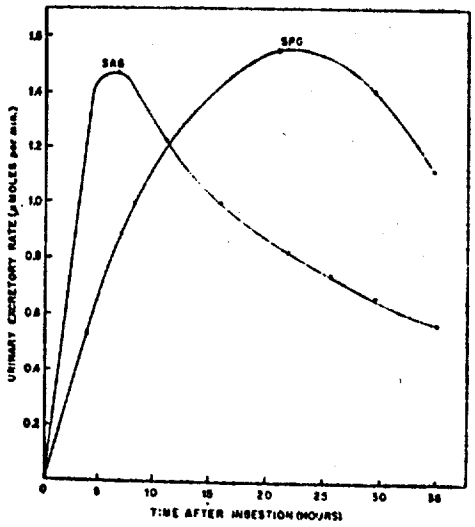

FIG. 5. RATES OF URINARY EXCRETION OF SAG AND SPG AFTER INGESTION OF SALICYLATE

the first, second, third, and fourth days, the cumulative urinary recoveries were 35.1, 59.1, 73.8, and 79.1 per cent. Consistently, 80 per cent appeared as the acyl glucuronide. The excretion of total hexuronic acid, estimated by the carbazole method, was equivalent to that of acyl glucuronide. By contrast, the urinary hexuronic acid concentration following benzoate was over three times that of benzoyl glucuronide, and after salicylate twice as much as that of the combined glucuronides. It thus seems likely that probenecid glucuronide is much less susceptible to hydrolysis in the body than the glucuronides of benzoate and salicylate.

DISCUSSION

Previous investigators have described the transfer to hydroxylamine of acyl groups linked to acids as anhydrides (2), or to alcohols as esters (17). It is of interest, therefore, that the acyl glucuronides, which constitute an intermediate category with acyl groups linked to a hemiacetal-bearing carbon, also form hydroxamates. This provides a sensitive method for their chemical estimation, and further characterizes them as a unique group within the class of glucuronides.

Conversion to the stable hydroxamates offers the advantage of a trapping mechanism for the readily hydrolyzed acyl glucuronides. These hydroxamates are readily extracted, purified, and subsequently identified. As a differential reaction, the hydroxamate method allows separate estimation of acyl and non-acyl glucuronides in mixtures of both. These several advantages made possible the observations described in this report.

Excretion of benzoyl glucuronide could be detected following the administration of as little as 6.9 millimoles of benzoate, although several times this amount has been required in the past (15). Thus it could be demonstrated that the glucuronide synthesis occurs independently, whether or not glycine conjugation is maximal. Only a minimal estimate of the actual rate of benzoyl glucuronide synthesis is obtained from the urinary recovery, as noted by Quick (15). The extent to which this conjugate may be broken down in the body is suggested by the three-fold excess of urinary hexuronic acid relative to benzoyl glucuronide. Therefore, it seems inappropriate to designate benzoyl glucuronide formation as merely a spare detoxication mechanism.

Following the oral administration of salicylate in this subject, three metabolites—salicylurate, SAG, and SPG—accounted for 90 per cent or more of the total urinary salicyl recovered. Other authors (6, 16) have reported the excretion of 20 to 25 per cent unconjugated salicylate under roughly comparable conditions, a difference possibly due to the breakdown of labile SAG, particularly in alkaline urines collected over prolonged periods. Kapp and Coburn (16) noted the urinary excretion of both acid- and alkaline-labile salicyl glucuronides,[7] and estimated their quantities. These authors suggested a diglucuronide structure for these conjugates, a possibility which the present results do not support. Of special interest was their subject W. J., who excreted equal amounts of the two glucuronides in the first 12 hours after intravenous salicylate, but almost twice as much acid-labile as alkaline-labile material in the succeeding 12 hours. These results clearly reflect the characteristic differences in the urinary excretory patterns of SAG and SPG described in the present studies.

SUMMARY

1. Acyl glucuronides react with hydroxylamine to form hydroxamic acids. This reaction may be applied to their estimation, purification, and identification.

2. Benzoate, salicylate, and probenecid elicited the urinary excretion of their respective acyl glucuronides when ingested orally by a normal subject.

3. Doses of sodium benzoate were quantitatively recovered as urinary hippurate and benzoyl glucuronide.

4. At least 90 per cent of various doses of salicylate was recovered as urinary salicylurate, salicyl acyl glucuronide, and salicyl phenolic glucuronide. Characteristic differences in the urinary excretory patterns of salicyl acyl glucuronide and salicyl phenolic glucuronide were described.

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[7] SAG is alkaline-labile, and SPG acid-labile.

J. Pharmacol. Exp. Therapeut. 126: 283-290, 1959.

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ABSORPTION OF DRUGS FROM THE RAT COLON[1]

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Received for publication March 20, 1959

It is well known that a number of substances are readily absorbed from the lower part of the intestinal tract. Thiopental and tribromoethanol are often administered by retention enema for the rapid induction of basal anesthesia. Salicylates administered rectally have been reported to give a satisfactory therapeutic response in patients with rheumatic fever (Parker, 1948). Paraldehyde has been administered rectally to produce obstetrical analgesia (Kotz and Kaufman, 1939). Quantitative studies have shown that phenol and ethanol are rapidly absorbed from colonic loops of the dog and cat (Sollmann *et al.*, 1910; Hanzlik and Collins, 1913). In contrast, poor colonic absorption has been reported for phenol red (Cooperstein and Brockman, 1959), glucose (McNealy and Willems, 1929), and sucrose (Curry and Bargen, 1935).

Because most of the studies have involved widely different techniques and indirect means of estimating the speed of absorption, it is difficult to recognize which properties of a drug govern its rate of absorption.

Previous work from this laboratory concerning absorption from the stomach and small intestine has demonstrated that the degree of ionization and lipid-solubility of a drug are important factors in determining its rate of absorption (Schanker *et al.*, 1957; Hogben *et al.*, 1957; Schanker *et al.*, 1958; Hogben *et al.*, 1959). Thus organic acids and bases are in general readily absorbed in their lipid-soluble, undissociated form while the ionized drug is absorbed with difficulty. Furthermore, unionized drugs with high lipid-solubilities are absorbed more rapidly than those with very low lipid-solubilities. The question arises as to whether these physical properties also govern the absorption of drugs from the colon.

The present report describes the colonic absorption of a large number of drugs in the rat. The results suggest that drugs are passively absorbed in the unionized form across a barrier which, like the blood:small intestine and blood:stomach barriers, is lipoidal in character.

METHODS AND MATERIALS. *Experimental procedure.* Male Sprague-Dawley rats weighing 110 to 150 g were fasted for about 20 hours prior to the experiments but were allowed free access to water. Coprophagy was prevented during the fasting period by housing the animals in cages having wide-mesh floors. Anesthesia was usually induced with pentobarbital and maintained with ether; however, when the absorption of barbiturates was investigated, ether was the sole anesthetic agent. The colon was exposed by a midline abdominal incision and was cannulated at its cecal end with polyethylene tubing having an outside diameter of 3.5 mm and a wall 1 mm thick. A similar cannula was then inserted into the rectum, to a distance of about 3 cm, where it was secured by a ligature. The length of colon between the 2 cannulae was about 13 cm in a 150 g rat. Perfusion of the colon *in situ*, from cecal to rectal ends, was begun after closing the abdominal incision.

A weakly buffered perfusion solution (pH 7.2) consisted of 1 mmol per l of drug together with the following salts in mmol per l: NaCl 145, KCl 4.56, $CaCl_2$ 1.25, Na_2HPO_4 1.33, and NaH_2PO_4 0.33. The concentrations of thiopental, phenylbutazone and hexethal were reduced to 0.5 mM because of their limited solubility in this solution. Several of the drugs studied were also perfused in a strongly buffered acidic solution which was prepared by adding aspartic acid (20 mmol per l) to the solution described above and adjusting the pH to 3.6 with HCl and NaOH. The sodium ion concentration of this solution was adjusted to 145 mM. The solutions were maintained at 37° C and were perfused with a Bowman infusion pump.

A. Absorption experiments: The colon was first cleared of particulate material by perfusion with the drug solution for 15 minutes at a rate of 1.5 ml per minute. The perfusion rate was then reduced to 0.2 ml per minute and the washing con

[1] A preliminary account of this work has appeared previously (THIS JOURNAL 122: 66A, 1958).

tinued for an additional 25 minutes following which four 20-minute samples were collected from the colonic outflow. The concentration of the drug was measured in each of the collected samples and the degree of absorption was calculated from the difference in the concentration entering and leaving the colon.

Inulin-C^{14} and phenol red, substances not absorbed, left the colon at a concentration 1.04 ± 0.02 times the concentration in the inflowing solution due to the absorption of water. Accordingly, the mean degrees of absorption of the various drugs were corrected by 4%. Direct evidence that the absorption of inulin-C^{14} and phenol red is negligible was obtained by placing 3 ml of saline solution containing known quantities of either substance in the ligated rat colon *in situ*. After 3 hours, 100 ± 3% of inulin and 101 ± 2% of phenol red were recovered by thoroughly washing the lumen with saline solution.

B. Steady state distribution of quinine between plasma and colonic lumen: Rats were prepared for perfusion of the colon *in situ* as described above. After washing the lumen with the drug solution for 10 minutes at a perfusion rate of 1.5 ml per minute, the perfusion rate was reduced to 0.2 ml per minute and quinine hydrochloride was slowly administered intravenously (50 mg/kg) during the next 10 minutes. Starting 20 minutes after the intravenous injection, 4 samples of the colonic outflow were collected at 10-minute intervals. For comparison of plasma and colonic quinine concentrations, a blood sample was taken immediately after collecting the 4th sample of the colonic outflow.

The composition of the perfusion solution (pH 7.2) was similar to that described for the absorption experiments except that the concentration of the drug was only 5.2 μg per ml. When this concentration of quinine was passed through the colon and the drug administered intravenously as described above, the concentration of the drug in the colonic outflow during the last 10 minutes of the experiment was the same as that of the inflowing solution. Thus a steady state was achieved since there was no net passage of the drug from gut to plasma or from plasma to gut.

In calculating the gut:plasma concentration ratio at the steady state, plasma levels were corrected for the degree of protein binding. The mean plasma level of quinine at the steady state was 3.6 μg per ml and the degree of protein binding was 80%.

C. The pH of solutions in the colon: Rats were prepared for perfusion of the colon *in situ* as described above. The lumen was washed with the perfusion solution for 20 minutes and the washings were discarded. The tubings attached to the inflow and outflow cannulae were then transferred to a flask containing 20 ml of fresh perfusion solution and this fluid was continuously circulated through the colon for 6 hours at a rate of 1.5 ml per minute. To prevent the extraneous exchange of carbon dioxide, the system was closed by a layer of liquid paraffin over the solution in the reservoir flask. Samples of the perfusion fluid were withdrawn anaerobically at various intervals and the pH was measured at 37° C under oil using a Beckman Model G pH meter. The compositions of the 3 solutions studied were: 1) Na^+ 160 mM, Cl^- 135 mM, HCO_3^- 25 mM; 2) NaCl 160 mM, saturated with 5% CO_2–95% O_2; 3) weakly buffered perfusion solution (pH 7.2) described for the absorption experiments.

Chemical and physical measurements. The analytical methods have been described or cited in previous publications (Schanker *et al.*, 1957, 1958; Hogben *et al.*, 1959) except for the following. 1) *o*- and *m*-Nitrophenol: samples were diluted with 0.1 M phosphate buffer (pH 11) and optical densities measured at 395 mμ. 2) Quinine was estimated fluorometrically according to the general method described by Brodie *et al.* (1947). Quinine was extracted from alkalinized aqueous samples into heptane containing 1.5% by volume of isoamyl alcohol. The drug was then returned to an aqueous phase of 0.1 N H_2SO_4 and the fluorescence measured. 3) Aprobarbital, phenobarbital, allylbarbituric acid, butethal, cyclobarbital, pentobarbital, hexethal, barbital and secobarbital were estimated according to the general method for barbiturates of Goldbaum (1948). 4) Inulin-C^{14} and mannitol-1,6-C^{14} were measured by the liquid counting technique of Cotlove (1955).

Chloroform:water partition coefficients of the undissociated forms of most of the drugs had been previously determined (Hogben *et al.*, 1959). Those of aprobarbital, phenobarbital, allylbarbituric acid, butethal, cyclobarbital, pentobarbital and hexethal were measured in exactly the same manner.

Dissociation constants for most of the drugs studied have been previously cited or determined (Schanker *et al.*, 1957, 1958). The dissociation constants of *o*- and *m* nitrophenol have been reported by Vandenbelt *et al.* (1951) and those of the oxybarbiturates studied were reported by Krahl (1940).

Binding of quinine to plasma proteins was measured by the ultrafiltration technique of Rehberg (1943).

Except where otherwise specified, ± will signify the range of values.

RESULTS. *Absorption of organic acids and bases.* The relative rates of absorption of acidic and

TABLE 1

Absorption of organic acids from the rat colon

The % absorbed is expressed as the mean ± the range. The figures in parentheses indicate the number of animals.

Acid	pKa	% Absorbed
5-Sulfosalicylic	(strong)	<2 (2)
Phenolsulfonphthalein	(strong)	<2 (7)
o-Nitrobenzoic	2.2	<2 (4)
5-Nitrosalicylic	2.3	2 ± 1 (4)
Salicylic	3.0	12 ± 2 (4)
m-Nitrobenzoic	3.4	10 ± 1 (4)
Benzoic	4.2	19 ± 4 (4)
Phenylbutazone	4.4	38 ± 3 (5)
o-Nitrophenol	7.0	55 ± 5 (6)
Thiopental	7.6	50*
p-Hydroxypropiophenone	7.8	46 ± 5 (4)
m-Nitrophenol	8.2	44 ± 5 (4)
Phenol	9.9	36 ± 3 (4)

* Standard deviation, ±10% for 30 experiments.

TABLE 2

Absorption of organic bases from the rat colon

The % absorbed is expresed as the mean ± the range. The figures in parentheses indicate the number of animals.

Base	pKa	% Absorbed
Acetanilide	0.3	36 ± 5 (4)
p-Nitroaniline	1.0	50 ± 8 (4)
Antipyrine	1.4	20 ± 2 (4)
m-Nitroaniline	2.5	48 ± 2 (3)
Aniline	4.6	44*
Aminopyrine	5.0	21 ± 2 (4)
p-Toluidine	5.3	51 ± 5 (4)
Quinine	8.4	20 ± 2 (4)
Levorphan	9.2	13 ± 1 (2)
Ephedrine	9.6	9 ± 2 (4)
Tolazoline (Priscoline)	10.3	4 ± 2 (3)
Mecamylamine (Inversine)	11.2	3 ± 2 (4)
Procaine amide ethobromide	(strong)	<2 (4)
Edrophonium (Tensilon)	(strong)	<2 (2)
Mepiperphenidol (Darstine)	(strong)	<2 (5)

* Standard deviation, ± 6% for 62 experiments.

basic drugs from the weakly buffered perfusion solution varied widely, ranging from less than 2% to as much as 55% after a single passage through the colon (tables 1 and 2). A relation between the degree of absorption and the dissociation constant[2] was revealed. The weak acids, those with a pKa above 4.3, were absorbed relatively rapidly (36 to 55%); the acids of intermediate strength, those with pKa values between 2.9 and 4.3, were absorbed more slowly (10 to 19%); and the strongest acids, those with a pKa below 2.4, were absorbed hardly at all. Similarly, the weak bases, those with a pKa below 8.5, were in general absorbed relatively rapidly (20 to 51%); the bases of intermediate strength, those with a pKa between 9 and 12, were absorbed more slowly (3 to 13%); and the completely ionized quaternary ammonium compounds were absorbed hardly at all.

[2] The dissociation constant of both acids and bases is expressed as a pKa which is the negative logarithm of the acidic dissociation constant. The pKa is defined by the Henderson-Hasselbalch equation:

$$\text{for acids.}\quad pKa = pH + \log \frac{[\text{unionized acid}]}{[\text{ionized acid}]}$$

$$\text{for bases.}\quad pKa = pH + \log \frac{[\text{ionized base}]}{[\text{unionized base}]}.$$

An acidic drug with a small pKa is a very strong acid and one with a large pKa a very weak acid. A base with a small pKa is a very weak base.

Effect of change in pH on absorption. The observation that the weak acids and bases were absorbed more readily than the stronger, highly ionized acids and bases suggests that the barrier separating the colonic lumen from blood is preferentially permeable to the unionized forms of drugs. From this viewpoint, the absorption of a drug would be expected to be enhanced by a change in pH that increased the proportion of the unionized drug moiety.

The effect of intralumenal pH on the absorption of several acidic and basic drugs is shown in table 3. The absorption of acidic drugs, which become more unionized at lower pH values, was markedly increased when the pH was lowered. For example, the degree of absorption of salicylic acid rose from 12% at pH 7 to 42% at pH 4. In contrast, the absorption of basic drugs, which become more ionized at lower pH values, was decreased when the pH was lowered. Thus quinine was absorbed to the extent of 20% at pH 7 and 9% at pH 4.

The very weak acid, phenol, which is almost

TABLE 3

Effect of intralumenal pH on absorption from the rat colon

The figures in parentheses indicate the number of animals.

Drug	pKa	pH of the Perfusion Solution	
		6.8–7.2*	3.6–4.0†
		% absorbed	*% absorbed*
Acid			
Salicylic	3.0	12	42 ± 3 (2)
Benzoic	4.2	19	50 ± 7 (2)
Phenol	9.9	36	37 ± 1 (2)
Base			
Aniline	4.6	44	32 ± 5 (14)
Quinine	8.4	20	9 ± 1 (2)

* This solution, which entered the colon with a pH of 7.2 and left with a pH of 6.8, is the weakly buffered saline solution described under METHODS. The absorption values have been taken from tables 1 and 2.

† This highly buffered solution entered the colon with a pH of 3.6 and left with a pH of 4.0.

completely unionized at both pH 7 and pH 4, showed no change in absorption when the pH was lowered.

Steady state distribution of quinine between plasma and colonic lumen. If the barrier separating the colonic lumen from plasma is selectively permeable to the undissociated form of a drug, the steady state distribution between plasma and colonic fluid will depend on the difference in pH of the fluids and the pKa of the drug. This relation can be expressed in the form of an equation which has been previously applied to the distribution of drugs across the gastric juice:blood barrier (Shore *et al.*, 1957) and the small intestine:blood barrier (Hogben *et al.*, 1959). Thus the theoretical steady state distribution of a weak base between the colonic lumen and plasma may be calculated from the following equation:

$$\frac{C_{colon}}{C_{plasma}} = \frac{1 + 10^{(pKa - pH_{colon})}}{1 + 10^{(pKa - pH_{plasma})}}.$$

Since the saline perfusate leaving the colon (pH 6.8) is more acidic than plasma, a base such as quinine (pKa 8.4) would be expected to reach a higher concentration within the colonic lumen than in plasma water when the steady state is achieved. Thus the predicted colon: plasma concentration ratio for quinine is 3.7.

The steady state distribution of quinine was determined experimentally in 3 animals by administering the drug intravenously and simultaneously perfusing it through the colon at a concentration which resulted in no net passage of drug from colon to plasma or from plasma to colon. The steady state concentration ratio, calculated by dividing the concentration of the colonic solution by that of plasma (corrected for plasma binding), was 7.2 ± 1.7.

Absorption and lipid-solubility of drugs. The studies presented thus far indicate that the uncharged drug molecule is, in general, readily absorbed while the ionized moiety is absorbed with difficulty. To determine whether the rates of absorption of undissociated molecules are related to their relative lipid-solubilities, the colonic absorption of 9 barbiturates was compared with the chloroform:water partition coefficients of the undissociated barbiturates. The variable of the degree of ionization of drugs was eliminated in this study since the compounds have pKa values between 7.4 and 8.1 (Krahl, 1940); accordingly they are largely undissociated at the pH (6.8) of the solution flowing through the colon. The results shown in table 4 reveal a fairly close relationship between absorption and the chloroform: water partition coefficient; in general, the greater the coefficient the greater the degree of absorption.

Additional evidence of the importance of lipid-solubility was obtained by determining the colonic absorption of 2 undissociated sub-

TABLE 4

Comparison between colonic absorption and lipid: water partition of the unionized forms of barbiturates

The % absorbed is expressed as the mean of 4 animals ± the range.

Barbiturate	% Absorbed	Chloroform to Water Partition Coefficient
Barbital	12 ± 2	0.7
Aprobarbital	17 ± 2	4.9
Phenobarbital	20 ± 3	4.8
Allylbarbituric acid	23 ± 3	10.5
Butethal	24 ± 3	11.7
Cyclobarbital	24 ± 2	13.9
Pentobarbital	30 ± 2	28.0
Secobarbital	40 ± 3	50.7
Hexethal	44 ± 3	>100

stances that are poorly lipid-soluble. Thus sulfaguanidine and mannitol, compounds having chloroform:water partition coefficients of less than 0.002, were absorbed to the extent of less than 3%.

Evidence for the passive nature of colonic absorption of drugs. If a substance is absorbed from the gut by a specialized transport mechanism, the process will approach saturation if the lumenal concentration is raised high enough (Höber, 1945). However, the colonic absorption of several acidic and basic compounds showed no evidence of saturation over a 10-fold range of concentration; rather, the absolute amount of drug absorbed was directly proportional to the initial concentration, the percentage absorption remaining constant (table 5). These results suggest that the colonic absorption of drugs is a matter of simple diffusion across the colonic epithelium.

The pH of solutions in the rat colon. The observation that the pH of the weakly buffered saline solution declined from 7.2 to 6.8 in passing through the colon seemed inconsistent with the generally accepted view that the contents of the colon are slightly alkaline. To investigate this matter more fully, a small volume of solution was continuously circulated through the colon for 6 hours and the pH measured at various times. The pH quickly declined from 7.2 to 6.8 and remained at this value throughout the remainder of the experiment (fig. 1, curve C).

TABLE 5

Effect of concentration on absorption from the colon

The % absorbed is expressed as the mean ± the range. The figures in parentheses indicate the number of animals.

Drug	Perfusion Solution Initial Concentration	% Absorbed
	mmol per l	
Salicylic acid	1	12 ± 2 (4)
	2	12 ± 3 (3)
	10	13 ± 2 (2)
Benzoic acid	1	19 ± 4 (4)
	2	18 ± 5 (4)
	10	19 ± 3 (2)
m-Nitrophenol	1	44 ± 5 (4)
	10	42 ± 4 (2)
Thiopental	0.05	48 ± 4 (6)
	0.5	50*
Aniline	1	44†
	10	43 ± 4 (2)
Quinine	1	20 ± 2 (4)
	10	20 ± 3 (2)

* Standard deviation, ±10% for 30 experiments.

† Standard deviation, ±6% for 62 experiments.

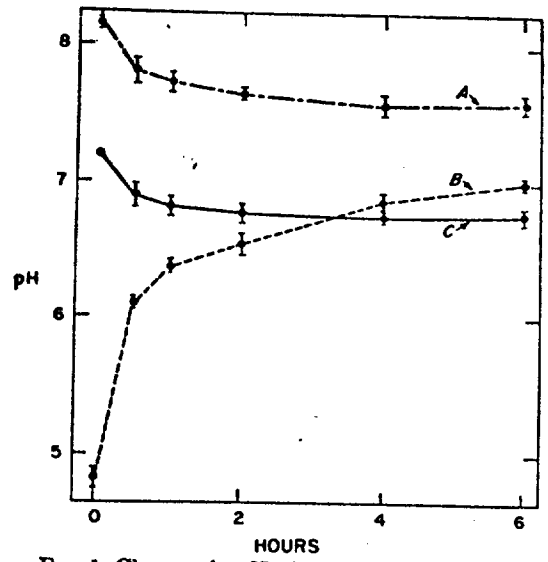

FIG. 1. Changes in pH of solutions continuously circulated through the rat colon.

Each curve represents the mean values and the range for 4 animals. Solution A) Na^+ 160 mM, Cl^- 135 mM, HCO_3^- 25 mM; Solution B) NaCl 160 mM, saturated with 5% CO_2–95% O_2; Solution C) the weakly buffered perfusion solution described under METHODS.

For comparison, 2 other isotonic solutions, one acidic (saline saturated with 5% CO_2) and one alkaline ($NaHCO_3$ and NaCl), were studied in the same manner. The pH of the acidic solution (fig. 1, curve B) gradually rose from about 4.8 and approached a value of about 7.0 after 6 hours. The pH of the alkaline solution (fig. 1, curve A) quickly declined from 8.3 to about 7.6 and remained at this value during the remainder of the experiment.

DISCUSSION. The rates of absorption of foreign organic electrolytes from the rat colon appear to be governed mainly by two physical properties: 1) their degree of ionization; and 2) the lipid: water partition of their undissociated forms.

The relation between the degree of ionization and rate of absorption of drugs is seen from the

relative absorption rates of a large number of compounds of widely different chemical structures. Thus weak acids with a pKa above 4.3 and weak bases with a pKa below 8.5 are in general readily absorbed;[3] stronger acids and bases are less readily absorbed, and highly ionized compounds are absorbed so poorly that the rates cannot be measured with the technique employed. This relation suggests that the colonic mucosa is selectively permeable to the uncharged drug molecules and that the ionized forms penetrate with difficulty. More direct evidence for this view is supplied by the changes in absorption of drugs that result from lowering the pH of the colon. Thus compounds which become more unionized are more readily absorbed while drugs which become more ionized are less readily absorbed.

The relationship between lipid-solubility and absorption is seen on comparing the rates of absorption of drugs and the lipid:water partition coefficients of their unionized forms. Thus all of the highly undissociated acids and bases, included in tables 1 and 2, which are rapidly absorbed, are readily extractable into fat-like solvents (Hogben *et al.*, 1959). In contrast, 2 lipid-insoluble substances, sulfaguanidine and mannitol, are very poorly absorbed despite their low degree of ionization. Further evidence of the importance of lipid-solubility is the close relationship between lipid:water partition ratio and the degree of absorption of a series of oxybarbiturates.

If the colonic absorption of drugs is simply a matter of diffusion of the unionized drug moiety across a lipoidal barrier, it should be possible to predict the steady state distribution of a drug across this boundary from knowledge of the pKa of the drug and the pH on either side of the boundary (Shore *et al.*, 1957). This relationship has been expressed as an equation under RESULTS. Thus the predicted colon:plasma steady state concentration ratio of quinine is 3.7, using the observed pH value of 6.8 for the colonic solution and pH 7.4 for plasma. In accord with this prediction, quinine is observed to be more concentrated within the colonic lumen than in plasma water when the steady state is achieved; however, the observed colon:plasma ratio is 7.2 rather than 3.7. The difference between the predicted and observed ratios may reflect a zone, at the site of absorption, that is only slightly more acidic than the measured pH (6.8) of the colonic solution.[4] Thus, substituting the observed ratio of 7.2 in the equation mentioned above, and solving for the colonic pH, we obtain a hypothetical or "virtual" colonic pH of 6.5. It is interesting to compare these results with a similar study of the rat small intestine (Hogben *et al.*, 1959). The steady state distributions of several drugs across the small intestinal epithelium indicate a "virtual" pH of about 5.3 which is distinctly more acidic than the pH (6.6) of the lumenal solution. A "virtual" pH of 5.3 for the small intestine and a value of 6.5 for the colon seem consistent with the observation of Parsons (1956) and others that solutions placed in the small intestine become decidedly more acidic than solutions placed in the colon.

Solutions in the rat colon do not become alkaline under the conditions of these experiments; rather, they tend to become neutral. Thus a saline solution (pH 7.2) weakly buffered with phosphate declined in pH to a constant value of 6.8 and a saline solution saturated with 5% CO_2 (pH 4.0 to 5.0) reached a pH value of only 7.0 after 6 hours of continuous circulation through

[3] There is an apparent upper limit of absorption of about 50% under the conditions of these experiments. Considerations of the perfusion technique, discussed at length in earlier publications (Schanker *et al.*, 1958; Hogben *et al.*, 1959), suggest that the most rapid rates of absorption are limited by the rate at which a drug can diffuse within the gut solution to reach the gut wall.

[4] The predicted colon:plasma ratio of 3.7 is based on the assumption that the epithelium is completely impermeable to the ionized moiety. However, the difference between the predicted and observed ratios would not be due to a very slight permeability to the ion in view of the following consideration.

In an earlier study of the distribution of weak organic electrolytes across the small intestinal epithelium (Hogben *et al.*, 1959) an equation was developed which allowed the calculation of the ratio of the permeability coefficients of the ionized and unionized moieties. Thus for an organic base, the steady state concentration ratio across the colonic epithelium can be written as:

$$\frac{C_{colon}}{C_{plasma}} = \frac{R + 10^{(pKa - pH_{plasma})}}{R + 10^{(pKa - pH_{colon})}} \cdot \frac{1 + 10^{(pKa - pH_{colon})}}{1 + 10^{(pKa - pH_{plasma})}}$$

where R is the ratio of the permeability coefficients (unionized/ionized).

Using the measured colonic pH of 6.8, the predicted colon:plasma concentration ratio of quinine would be 2.9 if R = 100, 3.6 if R = 1000, and 3.7 if the epithelium were completely impermeable to the ionized moiety.

the colon. Parsons (1956) reported that a saline solution buffered with bicarbonate (pH about 8.0) declined in pH to a value of about 7.5 when circulated through the rat colon. This finding has been confirmed in the present investigation. The failure of the bicarbonate-saline solution to decline in pH to a value as low as 6.8 to 7.0 is possibly explained by the recent findings of Cooperstein and Brockman (1959). Using the dog colon, these investigators found that even when there was a net absorption of bicarbonate, the lumenal concentration of bicarbonate increased as a result of the rapid absorption of water. D'Agostino *et al.* (1953) have also reported increasing bicarbonate concentrations in the dog colon, and Parsons (1956) demonstrated that bicarbonate becomes concentrated in the rat colon.

Many therapeutic agents are rapidly absorbed from the colon, but it is doubtful that this organ has an important role in the absorption of drugs administered by the oral route. The similarity in the patterns of drug absorption for the small and large intestines (Schanker *et al.*, 1957) makes it unlikely that a drug which had traversed the entire small intestine without being absorbed would be significantly absorbed in the colon. For the same reason, a drug that can be absorbed from the colon would have been completely absorbed in the small intestine.

The ready absorption of many drugs from the large intestine explains the effectiveness of rectal administration when the oral or parenteral routes of administration are undesirable. The rapid induction of basal anesthesia with rectally administered solutions of thiopental or tribromoethanol is an example of the effective use of the colon as a site of absorption. Truitt *et al.* (1950) found that administration of theophylline by retention enema resulted in higher blood levels compared to oral administration of the drug, but that absorption from a rectal suppository was very slow. Turell *et al.* (1940) showed that sulfanilamide was readily absorbed from aqueous solutions in the colon or rectum but poorly absorbed from suppositories. These observations indicate that for rapid absorption from the large intestine, a drug should be administered in aqueous solution so that it will exist in a readily diffusible form and be exposed to a large area of absorbing surface.

In the present study, several drugs were absorbed at rates too slow to be measured accurately. However, the results cannot necessarily be interpreted to mean that these compounds would be poorly absorbed in actual practice. In the present investigation, a solution of the drug was passed through the colon for a period of only 3 to 4 minutes contrasted with the much longer time that a drug might remain in the colon when administered rectally. On the other hand, the negligible absorption of the highly ionized compound, phenol red, is confirmed by the studies of Cooperstein and Brockman (1959).

While the stomach, small intestine and colon behave quite differently regarding the secretion and absorption of inorganic electrolytes (Hogben, 1955; Parsons, 1956; Cooperstein and Brockman, 1959), they are strikingly similar in regard to the absorption of foreign organic compounds (Schanker *et al.*, 1957, 1958). Weak acids and bases are readily absorbed when present in their lipid-soluble, undissociated form. Completely ionized drugs like quaternary ammonium compounds and sulfonic acids are poorly absorbed. Unionized substances that are lipid-insoluble are poorly absorbed. In the colon, stomach and small intestine, the relative rates of absorption of a number of drugs rank in similar fashion: thiopental > secobarbital > barbital; salicylic acid > acetylsalicylic acid. Indeed, the stomach, small intestine and colon may be pictured as segments of a canal that is bounded by a continuous lipoidal barrier, the qualitative variations in drug absorption in the different segments being mainly a function of the characteristic physiological pH gradients of each type of epithelium.

SUMMARY

The relative rates of absorption of a large number of acidic and basic drugs were measured in the colon of the anesthetized rat.

The degree of ionization and the lipid-solubility of organic electrolytes in solution are the most important factors determining their rates of absorption. Weak acids and bases were in general readily absorbed while stronger, highly ionized acids and bases were more slowly absorbed. The absorption of acidic drugs was increased and that of basic drugs decreased when the colonic contents were made more acidic. Weak organic electrolytes that are highly lipid-soluble were more readily absorbed than those which are poorly lipid-soluble.

The results suggest that many drugs are absorbed from the colon by simple diffusion of the unionized moiety across a lipoidal barrier.

The similarities of the patterns of absorption in the colon, small intestine and stomach indicate a common mechanism for the gastrointestinal absorption of drugs.

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ABSORPTION OF DRUGS FROM THE STOMACH I. THE RAT[1]

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Received for publication April 4, 1957

A number of investigations in the past have indicated that the stomach is capable of absorbing drugs. Karel, reviewing gastric absorption in 1948, was able to conclude only that "while the stomach is definitely not an absorptive organ in the same sense as the intestine and cannot be considered of especial importance in supplying the nutritional needs of the organism, its absorptive ability, particularly as regards substances physiologically active in minute quantities, has been grossly underestimated." The available fragmentary evidence does not provide a discernible pattern of absorption nor indicate the extent to which the stomach can absorb drugs.

The secretion of drugs into gastric juice is dependent on their ionization constants (Shore, Brodie and Hogben, 1957). This observation can be explained by postulating a lipoid barrier between plasma and gastric juice which allows passive transfer of drugs in their lipid-soluble unionized form, but not in their lipid-insoluble ionized form. On the basis of this model, a discernible pattern of gastric absorption would be expected; organic acids should be readily absorbed from acidic gastric juice while organic bases should not be appreciably absorbed.

The following report is a quantitative study of the absorption by the rat stomach of a large number of organic acids and bases having widely differing chemical structures, physical properties and pharmacologic activities. The observed rapid absorption of most acidic drugs and absence of absorption of most basic drugs will be interpreted in terms of the lipoid barrier hypothesis and the importance of the ionization constants.

METHODS AND MATERIALS. *Experimental procedure in rats.* Male Sprague-Dawley rats weighing 150 to 220 gm. were fasted for about twenty hours prior to the experiments. Water was given *ad libitum* and coprophagy was prevented by using cages with wide-mesh floors. The stomach was exposed under pentobarbital anesthesia and the cardia ligated. The pylorus was ligated around a short polyvinyl cannula equipped with a stopcock. Care was taken to exclude major blood vessels from the ligatures.

The gastric lumen was washed with saline until the washings were clear and then three times with drug solution warmed to body temperature. The drug solution was either 0.1 *M* HCl, 0.15 *M* NaCl or 0.15 *M* $NaHCO_3$ containing 200 microgm. of drug per ml. The abdominal incision was then closed. Ten ml. of the drug solution were drawn into a syringe and 1 ml. were introduced into the stomach and then withdrawn into the syringe. When this procedure had been repeated several times to insure thorough mixing, four to five ml. of the mixed drug solution (about 2.5 ml. per 100 gm. of body weight) were introduced into the stomach

[1] A preliminary report on this work was presented before the American Society for Pharmacology and Experimental Therapeutics, Charlottesville, Virginia, September 1954.

and the stopcock closed. The solution remaining in the syringe was called the initial solution. The drug solution was withdrawn from the stomach after 1 hour and this was called the final solution.

Phenol red or inulin-C^{14}, which were expected to be unabsorbed, were dissolved in the drug solution to indicate any volume change. After 1 hour, 98 ± 2 per cent of phenol red and 100 ± 2 per cent of inulin were recovered from the stomach whether these "indicators" were dissolved in the HCl, NaCl or $NaHCO_3$ solutions.

The concentrations of the drug and indicator were determined in the initial and final solutions. If C is concentration, the extent of drug absorption, appropriately corrected by the change of concentration of the indicator, is given by the following equation:

$$\text{Per cent absorbed} = 100 - 100\left(\frac{C_{\text{drug final}}}{C_{\text{drug initial}}} \times \frac{C_{\text{indicator initial}}}{C_{\text{indicator final}}}\right).$$

The magnitude of the correction due to dilution of phenol red or inulin was five to ten per cent.

The pH of the 0.1 *M* HCl drug solutions within the stomach remained constant at 1. The initial and final pH values of the $NaHCO_3$ solutions were between 8.0 and 8.3. The initially neutral saline solutions became distinctly acid (pH < 2) after 1 hour within the stomach because of the continuous secretion of acid by the rat stomach (Friedman, 1943).

Aniline secretion into the stomach, during D_2O and salicylic acid absorption, was determined by intravenously injecting 110 mgm. per kgm. of aniline. Fifteen minutes after this injection, the stomach was washed with a 0.1 *M* HCl solution of radioinulin, 10 per cent D_2O and 200 microgm. per ml. of sodium salicylate. The initial solution was obtained from the stomach as described above. The final solution was removed from the stomach at a specified interval. The difference in concentration of aniline between the final and initial solutions removed from the stomach was multiplied by the mean fluid volume within the stomach to give the amount of aniline secreted into the stomach. The mean plasma aniline concentration was approximated by correcting the terminal concentration for the rate of decline of plasma aniline, 15 per cent per 10 minutes.

Except where specified, ± will signify the range of values. No correction was made for body weight; inspection of the data did not suggest any bias attributable to this source.

Analytical methods. All spectrophotometric analyses were performed with a Beckman DU spectrophotometer.

A. Aniline, *m*-nitroaniline, *p*-toluidine and procaine amide ethobromide: A 0.1-ml. aliquot of the gastric solution containing 15 to 20 microgm. of the drug was diluted to 5 ml. with 0.1 *N* HCl and the drug determined by the Bratton and Marshall method (1939). Plasma aniline was determined according to the method of Brodie and Axelrod (1948).

B. Mecamylamine (3-methylaminoisocamphane): Five-tenths ml. of 2.5 *N* NaOH and 15 ml. of benzene[2] containing 1.5 per cent isoamyl alcohol[2] were added to a 1-ml. aqueous sample containing 5 to 50 microgm. of drug. The mixture was shaken for 15 minutes and then centrifuged. A 10-ml. aliquot of the benzene phase was shaken with 0.6 ml. of methyl orange reagent[3] for 5 minutes and the mixture was then centrifuged. Five ml. of the benzene phase were then transferred to a test tube containing 1 ml. of 2 per cent H_2SO_4 in absolute ethanol and optical density determined at 540 mμ. Standards were run concurrently with the unknown samples and the optical densities were proportional to concentration. An optical density of about 0.150 was obtained when 15 microgm. of mecamylamine hydrochloride were assayed by this procedure.

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[2] The solvents were purified by successive washings with 1 *N* NaOH, 1 *N* HCl and two washings with water.

[3] A stock solution of methyl orange reagent is made by dissolving 500 mgm. of methyl orange in 100 ml. of warm water. The solution is washed several times with chloroform. An aliquot of the resulting solution is diluted with an equal volume of saturated boric acid solution immediately before use.

C. Salicylic acid, benzoic acid and 5-nitrosalicylic acid: One-tenth ml. of concentrated HCl and 20 ml. of chloroform[2] were added to a 1- to 2-ml. aqueous sample of the drug. The mixture was shaken for 15 minutes and then centrifuged. A 15-ml. aliquot of the chloroform phase was shaken with 3 ml. of 0.1 *N* NaOH for 10 minutes. Two ml. of the aqueous phase were added to 1 ml. of 0.5 *N* HCl and the optical density of the resulting solution was determined at 300 mμ for salicylic acid, 273 mμ for benzoic acid or 310 mμ for 5-nitrosalicylic acid. Each drug was recovered with satisfactory precision: salicylate, 96 ± 3 per cent; benzoate 98 ± 3 per cent; 5-nitrosalicylate, 96 ± 3 per cent.

D. Acetylsalicylic acid or mixtures of acetylsalicylic acid and salicylic acid: Acetylsalicylic acid or mixtures of acetylsalicylic acid and salicylic acid were extracted into chloroform as described above for salicylic acid. The drugs were returned to an aqueous phase by shaking 15 ml. of this chloroform phase with 5 ml. of phosphate buffer (0.2 *M* Na_2HPO_4, pH 6.7) for 10 minutes. This buffer was chosen because acetylsalicylic acid would be rapidly hydrolyzed in an alkaline solution. Three ml. of the buffer phase were then added to 0.1 ml. of concentrated HCl and the optical density of the resulting solution was determined at 277 mμ and at 300 mμ. Since the ultraviolet absorption of acetylsalicylic acid at 300 mμ is negligible (less than 0.030 optical density at a concentration of 70 microgm. per ml.), the reading at 300 mμ is due to salicylic acid only. The corresponding optical density for salicylic acid at 277 mμ is read from a standard curve and is subtracted from the determined optical density at 277 mμ to give the value for acetylsalicylic acid alone. Recoveries of acetylsalicylic acid and salicylic acid from mixtures of the two were satisfactory: acetylsalicylic acid, 95 ± 3 per cent; salicylic acid, 96 ± 3 per cent.

E. 5-Sulfosalicylic acid: Aliquots of gastric solutions containing 5-sulfosalicylic acid were diluted with 20 to 30 volumes of 0.1 *N* HCl and the optical densities determined at 300 mμ. The blank due to biological material was negligible at these dilutions.

F. Phenol red: Samples of the gastric solution containing phenol red were appropriately diluted with 1 *N* NaOH and their optical densities determined at 550 mμ.

G. Isotopes: The Na^{22} content of liquid samples containing both radiosodium and radiochloride was measured with a well-type scintillation counter employing a glass vial thick enough to absorb Cl^{36} *beta* particles. These samples were then dried on planchets and counted with a thin window Geiger-Müller tube. The concentration of Na^{22} was subtracted from the total count measured by the Geiger-Müller tube to give the Cl^{36} content. The C^{14}-labeled materials, inulin and phenol, were measured by the liquid counting technique of Cotlove (1955). Total counts, several times background, exceeded 10,000 and the principle source of error was sample preparation (±2 per cent or less). Deuterium oxide samples were measured at the National Bureau of Standards (Broida *et al.*, 1954).[4]

H. Previously described methods: The following compounds were analyzed by previously described methods: *p*-hydroxypropiophenone (Shore *et al.*, 1957); acetanilide (Brodie and Axelrod, 1948); antipyrine (Brodie *et al.*, 1949); aminopyrine (Brodie and Axelrod, 1950); quinine (Josephson *et al.*, 1947); dextrorphan and levorphan (*d* and *l*-Dromoran) (Shore *et al.*, 1955); tolazoline (benzazoline, Priscoline) (Brodie *et al.*, 1952); thiopental (Brodie *et al.*, 1950); barbital and secobarbital (Goldbaum, 1948); caffeine (Axelrod and Reichenthal, 1953); tetraethylammonium (Mitchell and Clark, 1952); mepiperphenidol (Darstine) (Tillson *et al.*, 1954); *d*-α-acetylmethadol (Sung and Way, 1954); ephedrine (Axelrod, 1953); ethanol (Bucher and Rodetski, 1951).

Dissociation constants. The dissociation constants for mecamylamine and caffeine were determined by distribution between an organic solvent and 0.1 *M* aqueous buffers of various hydrogen ion concentrations as described by Butler (1953). Microtitration in 0.1 *M* saline was used for 5-nitrosalicylic acid and α-acetylmethadol. The spectrophotometric method of Flexser *et al.* (1935) was used for secobarbital. The following dissociation con-

[4] The authors wish to express their appreciation to Dr. Herbert Broida and Mrs. Margaret W. Chapman of the National Bureau of Standards for their invaluable help in the analysis of deuterium oxide

stants, determined in aqueous media, have been previously reported: aminopyrine, levorphan, acetanilide, quinine, antipyrine, thiopental, barbital, *p*-hydroxypropiophenone, tolazoline and salicylic acid (Shore *et al.*, 1957); benzoic acid, phenol, *m*-nitroaniline, aniline and *p*-toluidine (Vandenbelt *et al.*, 1954); ephedrine (Leffler *et al.*, 1951); acetylsalicylic acid (Edwards, 1950). All values are expressed as pKa.

The pKa of phenolsulfonphthalein (phenol red) is usually accepted in biological literature as 7.9. However, White and Acree (1919) clearly demonstrated that this compound, like other sulfonic acids, is a very strong acid. That is, phenolsulfonphthalein has a pKa_1 below 1.0 due to the strong sulfonic acid group; it also has a secondary pKa of 7.9 which is associated with the weak phenolic group. Therefore, phenolsulfonphthalein is almost completely ionized in solutions having a pH of 1.0 or above.

In vitro incubation. The entire stomach of a fasted, anesthetized rat was excised, split open along the greater curvature, weighed and incubated with 5 ml. of Krebs-Henseleit Ringer's bicarbonate solution containing 1 mgm. of drug. The incubation was carried out in a Dubnoff metabolic shaker for 1 hour at 37°C. in an atmosphere of 5 per cent CO_2-95 per cent O_2. Aliquots of the solution were analyzed for drug at zero time and after 1 hour of incubation. At the end of some experiments, the incubated stomach was homogenized and analyzed for drug content.

RESULTS. *Absorption from an acidic medium.* The gastric absorption of organic acids during one hour is shown in table 1. It is evident that, with the exception of barbital, those acids having pKa values greater than 2 were generally well absorbed (30 to 61 per cent) from 0.1 *M* HCl. On the other hand, the strong sulfonic acids, phenolsulfonphthalein and 5-sulfosalicylic acid, were not absorbed from this strongly acidic solution.

The gastric absorption values for organic bases are shown in table 2. All bases having pKa's greater than 2.5 were very poorly absorbed from 0.1 *M* HCl, the values being 0 to 7 per cent. Very weak bases (pKa < 2.5), however, were absorbed to the extent of 14 to 36 per cent, the weakest base having the largest absorption value.

TABLE 1

Absorption of organic acids from the rat stomach

The per cent absorbed in one hour is expressed as the mean ± the range followed by the number of experiments in parentheses.

Acid	pKa	Per cent absorbed in 1 hour		
		0.1 *M* HCl	Saline	$NaHCO_3$, pH 8
5-Sulfosalicylic	(strong)	0 ± 0 (2)	—	0 ± 0 (2)
Phenolsulfonphthalein	(strong)	2 ± 2 (3)	2 ± 1 (2)	2 ± 1 (2)
5-Nitrosalicylic	2.3	52 ± 3 (2)	—	16 ± 2 (2)
Salicylic	3.0	61 ± 7 (4)	35 ± 5 (4)	13 ± 1 (2)
Acetylsalicylic	3.5	35 ± 4 (3)	25 ± 2 (3)	—
Benzoic	4.2	55 ± 3 (2)	48 ± 3 (2)	—
Thiopental	7.6	46 ± 3 (2)	—	34 ± 2 (2)
p-Hydroxypropiophenone	7.8	55 ± 3 (2)	—	—
Barbital	7.8	4 ± 3 (4)	4 ± 3 (4)	—
Secobarbital	7.9	30 ± 2 (2)	—	—
Phenol	9.9	40 ± 5 (3)	—	40 ± 5 (3)

TABLE 2

Absorption of organic bases from the rat stomach

The per cent absorbed in one hour is expressed as the mean ± the range followed by the number of experiments in parentheses.

Base	pKa	Per cent absorbed in 1 hour		
		0.1 *M* HCl	Saline	$NaHCO_3$, pH 8
Acetanilide	0.3	36 ± 3 (2)	—	—
Caffeine	0.8	24 ± 3 (2)	—	—
Antipyrine	1.4	14 ± 3 (4)	22 ± 1 (2)	—
m-Nitroaniline	2.5	17 ± 0 (2)	—	—
Aniline	4.6	6 ± 4 (3)	12 ± 3 (2)	56 ± 3 (2)
Aminopyrine	5.0	2 ± 2 (3)	18 ± 3 (2)	—
p-Toluidine	5.3	0 ± 0 (2)	—	47 ± 4 (2)
α-Acetylmethadol	8.3	0 ± 0 (4)	—	—
Quinine	8.4	0 ± 0 (2)	7 ± 2 (2)	18 ± 2 (2)
Dextrorphan, Levorphan	9.2	0 ± 2 (8)	—	16 ± 1 (2)
Ephedrine	9.6	3 ± 3 (2)	—	—
Tolazoline	10.3	7 ± 2 (4)	—	—
Mecamylamine	11.2	0 ± 0 (2)	—	—
Darstine	(strong)	0 ± 0 (2)	3 ± 1 (2)	—
Procaine amide ethobromide	(strong)	0 ± 0 (2)	—	5 ± 1 (2)
Tetraethylammonium	(strong)	0 ± 1 (2)	—	—

Some early experiments indicated absorption of acetylmethadol and dextrorphan. These results have been omitted from table 2 because they could not be confirmed.

Absorption from an alkaline medium. The above results suggest that the unionized form of a drug is being absorbed. Because acidic drugs would be less unionized and basic drugs more unionized in an alkaline solution, absorption was measured from a $NaHCO_3$ solution. When acidic drugs having pKa values below 8 were dissolved in 0.15 *M* bicarbonate, absorption was significantly less than when they were dissolved in HCl (table 1). The depression was greatest for the stronger acids such as salicylic and 5-nitrosalicylic where the values dropped from 61 to 13 and from 52 to 16 per cent respectively. Depression of absorption was less marked for thiopental (pKa 7.6) and was not apparent at all in the case of phenol (pKa 9.9).

A marked increase in absorption was observed when bases were dissolved in the bicarbonate solution (table 2). This increase was greatest for the weaker bases such as aniline and *p*-toluidine where the absorption values rose from 6 to 56 and from 0 to 47 per cent respectively. The change in absorption was less marked for stronger bases such as quinine (pKa 8.4) and dextrorphan (pKa 9.2) whose values rose from 0 to about 17 per cent. The very strong base, procaine amide ethobromide, exhibited little if any increase in absorption.

Absorption from saline. When acidic and basic drugs were dissolved in normal saline, their absorption values were intermediate between those observed with the acidic and alkaline media (tables 1 and 2).

The pH of the saline solution, though initially near to neutral, became dis-

tinctly acid (pH < 2) after one hour within the stomach, but the rate at which the pH declined was certainly variable. Thus absorption in these experiments occurred from a solution whose pH, though poorly defined, was intermediate between the extremes of pH of the other experiments.

Absorption related to concentration. All absorption values shown in tables 1 and 2 were obtained using a concentration of 200 microgm. per ml. of drug. Several drugs were also studied at other concentrations: aminopyrine, 1000; antipyrine, 2000; barbital, 1000; dextrorphan, 400; salicylic acid, 100; secobarbital, 50; thiopental, 20 microgm. per ml. The per cent absorption of these drugs at the designated concentrations was the same as that observed at a concentration of 200 microgm. per ml. Since the per cent absorbed was constant, the absolute quantity of the drug absorbed was proportional to the concentration.

Absorption related to lipid solubility. Because the three barbituric acids are members of a homologous series, their relative rates of absorption have been compared to the lipid: water partition of their undissociated forms. The distribution of each acid between 0.1 *M* HCl and two lipid solvents, chloroform and heptane, is given in table 3. For this homologous series, there is a correlation between absorption and lipid solubility.

Absorption of one drug in the presence of another. In order to determine whether or not very high or very low absorption values were attributable to mucosal damage by the drug or to local vascular effects, a series of experiments was performed in which two drugs were studied simultaneously. If rapid absorption were due to mucosal damage, a poorly absorbed drug would be well absorbed in the presence of a rapidly absorbed drug. Conversely, if poor absorption resulted from local ischemia, a rapidly absorbed drug would be less well absorbed in the presence of a poorly absorbed drug. The data of table 4 clearly indicate that the presence of one drug had no significant effect on the absorption of the other, each being absorbed to its usual extent.

In vitro observations. Two techniques have been commonly employed to investigate gastric absorption. The determination of absorption by appearance of material in plasma is complicated by the intricate kinetics of disposition within the body. Determining the disappearance of a drug from the gastric lumen, the technique employed in this investigation, gives a direct measure of absorption provided that degradation and adsorption of the drug are negligible.

Metabolic degradation and adsorption were investigated *in vitro*. All drugs listed in tables 1 and 2 having absorption values greater than 5 per cent were incubated with the entire rat stomach for 1 hour. The concentration of all drugs, except aniline and *m*-nitroaniline[5], decreased by 9 to 13 per cent which is less than that expected from dilution in the water content of the stomach wall. Thus

[5] The following observations are not especially pertinent to this study of gastric absorption but they are of interest. The concentration of aniline and *m*-nitroaniline in the stomach wall, after incubation, was about twice that of the bathing solution. The total recovery of these drugs from the incubated stomach and the bathing solution was 95 per cent. The isolated frog stomach preferentially accumulates aniline as well as some other bases from the solution which bathes its serosal surface (Hogben, unpublished observations). During absorption *in vivo*, such an accumulation of drug within the epithelium would be restricted by the low plasma concentration of the drug.

determining drug absorption by disappearance from the gastric lumen is a valid technique.

Gastric blood flow during absorption. The possibility that gastric blood flow might limit those rates of absorption which are most rapid was examined by simultaneously comparing the absorption of D_2O and salicylic acid from the stomach with the secretion of aniline into the stomach. The results are presented in table 5. After aniline had been injected intravenously, the 0.1 *M* HCl solution of D_2O, salicylic acid and radioinulin was introduced into the stomach. In these experiments, the absorption period and the volume introduced into the stomach were varied as indicated. The mean per cent absorption of D_2O and salicylic acid was respectively 33 and 30 in 20 minutes and 48 and 45 in 40 to 50 minutes.

In order to undertake a comparison of absorption and secretion, it is desirable to express the results in terms of clearance. The amount of aniline which is secreted into the stomach (microgm. per hr.) divided by the mean plasma concentration (microgm. per ml.) is the gastric clearance of aniline (ml. per hr.). Aniline clearance must represent the minimal gastric mucosal blood flow and may be a very close estimate (Shore *et al.*, 1957).

In order to calculate the rate of absorption as a clearance, it is necessary to correct for the change of concentration that occurs during absorption. This correction is applied in the following equation:[6]

$$\text{Clearance} = \frac{\text{Volume}}{\text{time}} \cdot \ln\left(\frac{\text{initial concentration}}{\text{final concentration}}\right).$$

The clearances of D_2O and salicylic acid were calculated using the mean volumes within the stomach and the concentration changes corrected for radioinulin dilution.

Clearly blood flow plays only a minor role in limiting absorption as the gastric mucosal blood flow is at least 10 times greater than the most rapid rate of absorption that we have observed (table 5).

[6] The following assumptions can be applied to gastric absorption (Berggren and Goldberg, 1940). The rate of absorption is proportional to the concentration gradient across the mucosa. During most of the period of absorption, the plasma concentration will be insignificant. Then, the amount disappearing from the stomach, $\frac{dc}{dt} \cdot V$, will be proportional to the concentration within the stomach, or

$$-\frac{dc}{dt} \cdot V = \alpha \cdot c$$

where c = concentration, t = time, V = volume and α is the proportionality constant or the clearance factor. On integration this yields:

$$\ln c = -\frac{t}{V}\alpha + k$$

The integration constant, k, can be substituted using c = initial c at t = 0 to give the equation presented in the text. This equation should be considered only a good working approximation. Integration of diffusion equations are usually more complex, *e.g.* for diffusion out of a sphere.

TABLE 3

Comparison between gastric absorption of barbiturates and lipid:water partition of the undissociated barbiturates

Each compound was distributed between equal volumes of 0.1 *M* HCl and an organic solvent. The concentration of barbiturate in the organic phase divided by the concentration of barbiturate in the aqueous phase is expressed as K.

Barbiturate	pKa	Per cent absorbed from 0.1 *M* HCl	$K_{chloroform}$	$K_{heptane}$
Barbital	7.8	4	0.7	<0.001
Secobarbital	7.9	30	23.3	0.10
Thiopental	7.6	46	>100.0	3.30

TABLE 4

Gastric absorption of one drug in the presence of a second drug

Drug 1	Drug 2	Per cent absorbed from 0.1 *M* HCl in 1 hour			
		Drug 1		Drug 2	
		Alone	In presence of drug 2	Alone	In presence of drug 1
Dextrorphan	Phenol	0	0	40	38
Dextrorphan	5-Nitrosalicylic acid	0	0	52	55
Phenol	Acetylmethadol	40	40	0	0
Acetylsalicylic acid	Salicylic acid	35	38	61	60
Aniline	Salicylic acid	6	0	61	53

TABLE 5

Relative gastric clearance of aniline, deuterium oxide and salicylic acid by the rat

The absorption from the stomach of D_2O and salicylic acid and the secretion of aniline into the stomach were measured simultaneously. These results have been expressed as clearance. The procedure for calculating clearance is given in the text.

Rat	Weight	Time interval	Volume recovered	Inulin ratio $\frac{\text{final}}{\text{initial}}$	Terminal plasma aniline	Clearance *ml. per hour*		
						D_2O	Salicylic acid	Aniline
	gm.	*min.*	*ml.*		*microgm. per ml.*			
1	276	20	3.8	0.96	94	5.3	4.7	70
2	244	20	4.3	0.96	86	4.1	3.0	59
3	233	20	3.8	0.94	85	4.7	4.5	78
4	282	40	7.6	0.94	66	8.4	6.5	98
5	246	40	6.8	0.94	67	5.3	6.2	63
6	235	50	6.8	0.96	60	5.2	4.7	78
Mean	253					5.5	4.9	74

Absorption of other substances. For comparative purposes, gastric absorption was determined for several materials that are not weak electrolytes. Ethanol, in a concentration of 200 microgm. per ml., was absorbed from 0.1 *M* HCl to the extent of 25 ± 2 per cent in 2 animals. The unidirectional movement, *i.e.* flux,

from gastric lumen to blood was determined for water, sodium and chloride by measuring the disappearance of the respective isotopes from the gastric lumen during 1 hour. A normal saline solution of D_2O, 10 per cent by volume, lost heavy water to the extent of 61 ± 2 per cent in 2 animals whose mean weight was 116 gm. The change of concentration of Na^{22} and Cl^{36} was determined with the isotopes incorporated in a solution having an ionic composition of Na^+ 1 *mM*, Cl^- 101 *mM* and H^+ 100 *mM*. Phenol red was used as the dilution indicator. In 10 animals, mean weight 154 ± 7 gm., the per cent decrease in concentration of Na^{22} was 2.2 per cent ± 1.3 (standard error of mean). Cl^{36} disappeared to the extent of 14.4 per cent ± 2.7 (standard error of mean) in 6 animals whose mean weight was 160 ± 12 gm.

Discussion. The absorption of drugs by the rat stomach is recognizably determined by the pKa of the drug. The relative concentrations of the undissociated and the dissociated forms of a drug are given by the Henderson-Hasselbach equation:

$$\text{pH} = \text{pKa} + \log \frac{[\text{unionized basic drug}]}{[\text{ionized basic drug}]}$$

$$\text{pH} = \text{pKa} + \log \frac{[\text{ionized acidic drug}]}{[\text{unionized acidic drug}]}.$$

The rapid absorption from an acidic medium of those acids whose pKa is greater than 2, the absence of absorption of two very strong acids and the negligible absorption of all bases except those that are very weak (pKa < 2.5), are clearly consistent with the hypothesis that the gastric mucosa is selectively permeable to the undissociated form of a drug. When the pH of the gastric lumen was 1.0, appreciable absorption only occurred if a drug was largely undissociated.

The concept of the gastric mucosa as a barrier selectively permeable to the undissociated form of a drug was formulated by Travell in 1940. She was able to show that absorption of a base such as strychnine would occur from an alkaline solution while absorption was difficult to demonstrate from the normally acid contents of the stomach. Previously, Bradley *et al.*, (1936) had found that the absorption of salicylic and acetylsalicylic acids from the dog stomach was greatest at lower pH values.

If the gastric mucosa were selectively permeable to the undissociated drug, increasing the pH within the gastric lumen should depress absorption of an acid and facilitate absorption of a base. The contrast between absorption from solutions of pH 1 and 8 confirms this expectation. Absorption from initially neutral saline was intermediate between absorption at these two extremes of pH.

We have assumed that gastric absorption of drugs is simply a matter of passive diffusion of the unionized moiety. If this were so, absorption should be proportional to concentration, or the per cent absorbed independent of concentration. In those instances where absorption was measured at two different concentrations, the per cent absorbed did not change. Thus, it is not necessary to postulate specific transport mechanisms to account for the gastric absorption of drugs.

The evidence considered so far supports only the contention that the gastric

mucosa is selectively permeable to undissociated weak electrolytes. We would like to go further and suggest that this selective permeability is attributable to a lipoid character of the gastric membrane. The striking difference in absorption of the three barbituric acids correlates with their lipid:water partition coefficients. The only exception encountered to a generalization that all but the strongest acids are rapidly absorbed is barbital. Its poor absorption can be explained by its lipid insolubility. Ethanol is absorbed significantly more slowly than most of the weak acids even though it has a smaller molecular volume. This can be attributed to the more polar nature of ethanol. Thus, the gastric absorptive surface is similar to many other biological membranes which are considered to have a lipoid structure (Höber, 1945).

The pH at the interface across which absorption occurs is well defined when the pH of the bulk solution within ths stomach is one. When the bulk solution is less acid, the pH within the tubules of the gastric mucosa will be poorly defined due to the continuous secretion of acid by the rat stomach (Friedman, 1943). Consequently, when the pH of the solution within the stomach is varied, the rate of drug absorption will not be quantitatively proportional to the concentration of the unionized moiety in this solution. This is illustrated by a salicylic acid absorption of 61 per cent from HCl and 13 per cent from $NaHCO_3$ despite a 10^6 difference in the concentration of unionized salicylic acid.

While the predominantly lipoid character of the gastric mucosa is most important for drug absorption, the gastric mucosa is not an exception to Collander and Bärlund's lipoid-sieve model of cell membranes. The observed rapid rate of deuterium oxide movement can be attributed to small holes, not much larger than the diameter of the water molecule, which puncture the much more extensive lipoid area. Such aqueous pores would account for the slow escape of radiosodium and the disappearance of radiochloride from the gastric cavity, though chloride also moves by active transport (Hogben, 1955). The passage of larger organic ions through narrow pores would occur at a much slower rate and our present technique did not demonstrate absorption of either very strong acids or bases which are essentially completely ionized. This relative impermeability to organic ions is also confirmed by the concentration of drugs secreted into gastric juice (Shore *et al.*, 1957).

Finally, it is pertinent to consider the upper limit of absorption exhibited by D_2O and many of the weak acids, namely 50 to 60 per cent from 5 ml. in one hour. One of the following factors must impose this limit on absorption: the restraint imposed by the mucosa; the rate of gastric mucosal blood flow; or diffusion within the lumen. The experiments contrasting clearance of D_2O, salicylic acid and aniline clearly indicate that gastric mucosal blood flow is not the limiting step.[7] Until more is known, it is necessary to consider that the highest rates of

[7] The gastric absorption of D_2O by the dog and the human subject is apparently not blood-flow limited. The clearance of D_2O by the canine gastric pouch approximates the rate of gastric juice secretion (Cope *et al.*, 1943) but the clearance of aniline into the stomach is forty times greater (Shore *et al.*, 1957). The human D_2O clearance is not much more rapid than the normal maximum secretory rate (Hogben *et al.*, 1957) while the basic dye neutral red is much more concentrated in gastric juice than it is in plasma (Kolm *et al.*, 1945).

absorption obtained in this study may be only an indication of diffusion and mixing within the stomach lumen and that the gastric epithelium is no longer the limiting factor.

The rat stomach is recognizably different from the human or dog stomach in at least one important respect. The proximal third of the rat stomach is lined not by typical gastric mucosa but by keratinized squamous epithelium. In spite of this anatomical difference, absorption from the rat stomach is both qualitatively and quantitatively similar to absorption from the human stomach (Hogben *et al.*, 1957).

SUMMARY

When gastric contents are acid, the rat stomach rapidly absorbs most acidic drugs. In contrast, all but the very weakest basic drugs are poorly absorbed. When gastric contents are alkaline, absorption of acidic drugs is impeded and that of basic drugs facilitated. These observations have been presented in terms of the hypothesis that the gastric mucosa is predominantly a lipoid barrier. Gastric absorption of drugs can be explained by the diffusion of the unionized form of a weak electrolyte across such a barrier.

The unionized forms of drugs traverse the mucosa at rates approaching deuterium oxide movement and usually more rapidly than ethanol.

Rapid absorption by the stomach is not limited by gastric mucosal blood flow. When absorption is rapid, diffusion and mixing within the gastric lumen may become the limiting factors.

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J. Pharmacol. Exp. Therapeut. 123: 81-88, 1958.

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ABSORPTION OF DRUGS FROM THE RAT SMALL INTESTINE[1]

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Received for publication January 15, 1958

An understanding of the factors that govern the intestinal absorption of foreign compounds would furnish a useful guideline for predicting the rapidity and degree of absorption of a new drug. While the literature abounds with reports of the absorption of drugs, the nature of the experiments is such that a clear pattern of how drugs are absorbed is not discernible. Many of the studies involve indirect measurements, appraising intestinal absorption by the rate of appearance of drug in plasma, the excretion in urine, or by the speed of onset or degree of pharmacologic action. This information, though valuable in judging the absorption of a particular drug, has not yielded a generalized picture because of the variables of drug distribution, metabolism and urinary excretion. In more direct studies, absorption has been measured by the disappearance of a drug from the gut. In general, these are concerned with absorption from an intestinal loop *in situ*. Occasionally they deal with the absorption of several closely related compounds but more often with only a single compound, for example, choline (Rohse and Searle, 1955), Dromoran (Fisher and Long, 1953), mecamylamine (Zawoiski *et al.*, 1956) and salicylic and acetylsalicylic acids (Bradley *et al*, 1936). The widely different experimental conditions used in the various studies make it difficult to recognize the properties of drugs that govern their absorption.

Previous studies in our laboratory have shown that the boundary between plasma and the gastric lumen allows the passage of drugs in their lipid-soluble unionized form but is resistant to the penetration of the ionic form. Accordingly the dissociation constant of a drug is one of the most important characteristics which determines its passage across the gastric mucosa. The concept of a lipoid membrane between plasma and gastric lumen permits the prediction of the extent to which basic drugs are concentrated in the gastric juice after their intravenous administration and also the prediction that acidic drugs are absorbed directly from the stomach (Shore *et al.*, 1957; Schanker *et al.*, 1957; Hogben *et al.*, 1957b).

The present report describes the absorption of a large number of acidic and basic compounds by the rat small intestine. The results, which indicate that the dissociation constant is an important factor in determining the intestinal absorption of drugs, are compatible with the concept that drugs are passively absorbed in the unionized form, possibly across a lipoid barrier.

METHODS AND MATERIALS. *Experimental procedure.* Male Sprague-Dawley rats weighing 120 to 200 gm. were fasted for about 20 hours prior to the experiments but water was allowed *ad libitum*. During the fasting period, the animals were kept in cages having wide-mesh floors to prevent coprophagy. The animals were anesthetized with pentobarbital and the small intestine exposed by a midline abdominal incision. The intestine was cannulated at the duodenal and ileal ends with polyethylene cannulae having inside diameters of 2.5 mm. and outside diameters of 3.5 mm. The stomach and cecum were closed off by ligatures, care being taken not to occlude major blood vessels. In most of the experiments, the bile duct was ligated. The intestine was replaced in the abdomen, the incision closed and perfusion, from pylorus to ileocecal junction, started immediately.

The perfusion solution (pH 7.2) consisted of 1 *mM* per l. of drug together with the following salts in *mM* per l.: NaCl 145, KCl 4.56, $CaCl_2$ 1.25, Na_2HPO_4 1.33, and NaH_2PO_4 0.33. The solution was maintained at 37° C. and was perfused with a Bowman infusion pump at a rate of 1.5 ml. per

[1] A preliminary account of this work has appeared previously, Fed. Proc., 15: 480, 1956.

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minute. A 1-ml. graduated pipette, placed vertically in the perfusion system by means of a T-tube, indicated changes in intralumenal pressure. After passage of the fluid through the intestine, the pH of the outflow, collected under oil, was 6.6.

A. Single perfusion technique: The small intestine was first cleared of particulate matter by perfusion with a drug-free solution for 30 minutes. The solution containing drug was then perfused for 30 minutes to displace the previous wash, after which four 10-minute samples were collected from the ileal outflow. The concentration of drug was measured in each of the collected samples and the relative rate of absorption was calculated from the difference in the concentration entering and leaving the intestine.

The necessity for perfusing the intestine with drug solution for 30 minutes before measuring drug absorption was established by showing that the outflowing concentration of inulin-C^{14}, a substance which was not absorbed, did not become constant until after 25 minutes of perfusion. This steady state concentration of inulin was 1.03 ± 0.02 times the concentration in the inflowing solution indicating a small decrease in the volume of the perfusion fluid. This volume change, due to the absorption of water, was determined in a large number of experiments by actual measurement of the rates of inflow and outflow. The mean decrease in volume was 3 per cent which agreed with the inulin concentration ratio of 1.03. Consequently, the mean values of the relative change in drug concentration were corrected by 3 per cent. As indicated below, the absorption of inulin is insignificant.

B. Recirculation technique: Drugs whose absorption rates were slow when measured by the above procedure were also studied by a recirculation technique which exaggerated the decrease in drug concentration. The intestine was first perfused for 30 minutes with drug solution containing either inulin-C^{14} or phenol red. The tubings attached to the inflow and outflow cannulae were then transferred to a flask containing 30 ml. of drug solution. This volume was then continuously circulated through the small intestine for 3 hours. The relative rate of absorption was calculated from the decrease in drug concentration, corrected for the volume change as follows:

Per cent absorbed

$$= 100 - 100\left(\frac{C_{drug\ final}}{C_{drug\ initial}} \times \frac{C_{indicator\ initial}}{C_{indicator\ final}}\right).$$

After recirculation for 3 hours, the mean concentration of the volume change indicator, inulin or phenol red, was 1.20 ± 0.11 times the initial concentration. When known quantities of these same substances were circulated through the intestine for 3 hours and the intestine then thoroughly washed with saline, 97 ± 2 per cent of the inulin and 98 ± 2 per cent of the phenol red were recovered. Thus the negligible absorption of inulin and phenol red permits their use as indicators of volume change.

Unless otherwise specified, ± will refer to the range of values.

Analytical methods. The analytical methods have been described or cited in a previous publication (Schanker *et al.*, 1957)[3] except for the following. A. Tensilon (3-hydroxyphenyldimethylethyl ammonium chloride): Samples were diluted with 0.1 *N* NaOH to give a final concentration of 5 to 80 microgm. per ml. Optical densities were measured at 295 mμ. B. *m*-Nitrobenzoic acid: Samples were diluted with 1 *N* HCl to give a final concentration of 3 to 40 microgm. per ml. of the drug. Optical densities were measured at 2[illegible] mμ. C. *o*-Nitrobenzoic acid: Samples were diluted with 3 *N* HCl so that the final concentration of drug was 3 to 50 microgm. per ml. Optical densities were measured at 270 mμ. D. Bromphenol blue (tetrabromophenolsulfonphthalein): Samples containing bromphenol blue were diluted with 1.7 *M* K_2HPO_4 and the optical densities measured at 600 mμ. E. Isotopes: Acetic acid-1-C^{14}, phenol-1-C^{14}, urea-C^{14} and inulin-C^{14} were measured by the liquid counting technique of Cotlove (1955). F. Tromexan (ethyl biscoumacetate) was estimated according to Brodie *et al.* (1952a), phenylbutazone according to Burns *et al.* (1953) and theophylline according to Brodie *et al.* (1952b).

Dissociation constants. The dissociation constant for Tromexan was determined by distribution of the drug between heptane and various 0.1 *M* aqueous buffers as described by Butler (1953). The dissociation constants of theophylline and phenylbutazone have been reported by Shore *et al.* (1957), and those of *p*-nitroaniline, *o*-nitrobenzoic acid and *m*-nitrobenzoic acid by Vandenbelt *et al.* (1954). The dissociation constants of other compounds studied were cited in a previous publication (Schanker *et al.*, 1957).

Drug Binding. The binding of drugs to nondialyzable substances in the intestinal perfusate was measured by the ultrafiltration technique of Rehberg (1943).

RESULTS. The reproducibility of the values obtained with the single perfusion technique was investigated by measuring the absorption of a

[3] The authors wish to express their appreciation to Dr. Herbert Broida and Mrs. Margaret W. Chapman of the National Bureau of Standards for their invaluable help in the analysis of deuterium oxide.

physical acid and base. Salicylic acid was absorbed to the extent of 60 per cent (standard deviation ± 10 per cent) in 30 animals; aniline was absorbed 54 per cent (standard deviation ± 10 per cent) in 43 animals.

Absorption from various segments of the small intestine. The absorption of salicylic acid and aniline from different segments of the small intestine was measured and compared with that of the entire small intestine. The intestine was divided into various segments and each was perfused simultaneously with the drug solution. The results are presented in table 1. The absorption from each segment was proportional to its length and the summated absorption of the segments equaled that of the entire small intestine. Thus, in spite of anatomical and physiological differences in various parts of the small intestine, absorption was similar in the upper, middle and lower one-thirds.

Failure to find evidence for specific transport mechanisms. In looking for evidence that drugs might be absorbed by specific processes, the absorption of salicylic acid and aniline was measured at various concentrations. The proportion of salicylic acid and aniline absorbed was found to be constant over a wide concentration range; that is, the amount absorbed was directly proportional to the concentration (table 2). The failure to find evidence of saturation of the absorption process over a wide concentration range suggests, but does not prove, that salicylic acid and aniline are absorbed by a physical process.

Further indirect evidence of a physical process was seen in the failure of one drug to alter the absorption of another. When solutions of the following pairs of acids or bases were perfused, each drug was absorbed as though it were present alone: salicylic acid and acetylsalicylic acid, quinine and aniline, aminopyrine and aniline, *p*-toluidine and Tensilon, and *p*-hydroxypropiophenone and quinine.

It is also noteworthy that the presence of aniline did not modify the absorption of any of the acidic drugs studied nor did salicylic acid modify the absorption of any basic drug. Consequently these two compounds were selected as "internal standards." When the absorption of an acidic drug was being measured, aniline absorption was measured simultaneously; when a basic drug was studied, salicylic acid absorption was simultaneously measured. The observed rates of absorption of a given drug could then be corrected for variation among individual animals on the basis of the deviation from the mean rate of absorption of the reference material, aniline or salicylic acid.

Absorption of drugs after a single perfusion through the small intestine. The relative rates of absorption of a considerable number of acidic and basic drugs were determined. A relation between the dissociation constant and the degree of absorption was evident (tables 3 and 4). Most

TABLE 1

Absorption of drugs from various segments of the rat small intestine

Absorption was initially determined for the entire length of small intestine. The intestine was then divided into 2 portions and absorption measured in each simultaneously. The results are the mean values from 6 experiments.

Intestinal Segment	Per Cent Absorbed	
	Salicylic acid	Aniline
Entire length	55	51
Upper one-third	20	19
Lower two-thirds	33	30
Entire length	61	55
Upper two-thirds	40	37
Lower one-third	18	16

TABLE 2

Effect of concentration on intestinal absorption

The per cent absorbed is expressed as the mean ± the range followed by the number of experiments in parentheses.

Drug	Perfusion Solution Initial Concentration	Per Cent Absorbed
	mM per l.	
Salicylic acid	0.5	53 ± 4 (4)
	1.0	60*
	10.0	54 ± 5 (4)
Aniline	0.1	58 ± 5 (4)
	1.0	54†
	10.0	59 ± 4 (4)
	20.0	54 ± 5 (3)

* Standard deviation, ±10 per cent for 30 experiments.

† Standard deviation, ±10 per cent for 43 experiments.

TABLE 3

Absorption of organic acids from the rat small intestine

The per cent absorbed is expressed as the mean ± the range followed by the number of experiments in parentheses.

Acid	pK_a	Per Cent Absorbed	
		Actual	Relative to aniline
5-Sulfosalicylic	(strong)	<2 (2)	<2
Phenol red	(strong)	<2 (4)	<2
Bromphenol blue	(strong)	<2 (2)	<2
o-Nitrobenzoic	2.2	5 ± 2 (2)	5
5-Nitrosalicylic	2.3	9 ± 2 (3)	9
Tromexan	2.9	35 ± 7 (3)	37
Salicylic	3.0	60*	—
m-Nitrobenzoic	3.4	53 ± 0 (2)	50
Acetylsalicylic	3.5	20 ± 4 (6)	21
Benzoic	4.2	51 ± 5 (2)	54
Phenylbutazone	4.4	65 ± 7 (3)	54
Acetic	4.7	42 ± 1 (3)	40
Thiopental	7.6	55 ± 6 (3)	67
Barbital	7.8	30 ± 4 (2)	25
p-Hydroxypropiophenone	7.8	61 ± 9 (5)	61
Phenol	9.9	51 ± 8 (3)	60

* Standard deviation, ±10 per cent for 30 experiments.

TABLE 4

Absorption of organic bases from the rat small intestine

The per cent absorbed is expressed as the mean ± the range followed by the number of experiments in parentheses.

Base	pK_a	Per Cent Absorbed	
		Actual	Relative to salicylic acid
Acetanilide	0.3	42 ± 5 (2)	43
Theophylline	0.7	29 ± 1 (3)	30
p-Nitroaniline	1.0	68 ± 7 (2)	61
Antipyrine	1.4	32 ± 6 (3)	30
m-Nitroaniline	2.5	77 ± 2 (2)	63
Aniline	4.6	54*	—
Aminopyrine	5.0	33 ± 4 (4)	27
p-Toluidine	5.3	59 ± 3 (3)	56
Quinine	8.4	15 ± 2 (6)	15
Ephedrine	9.6	7 ± 3 (2)	6
Tolazoline	10.3	6 ± 1 (2)	5
Mecamylamine	11.2	<2 (2)	<2
Darstine	(strong)	<2 (2)	<2
Procaine amide ethobromide	(strong)	<2 (2)	<2
Tetraethylammonium	(strong)	<2 (2)	<2
Tensilon	(strong)	<2 (2)	<2

* Standard deviation, ±10 per cent for 43 experiments.

of the drugs were readily absorbed except for the stronger acids and bases. The stronger acids with a pK_a below 2.9 were slowly absorbed (less than 9 per cent), while the weaker acids with a pK_a greater than 2.9 were generally rapidly absorbed (21 to 67 per cent). The weaker bases with a pK_a less than 8 were rapidly absorbed (27 to 63 per cent), while the stronger bases with a pK_a greater than 9 were slowly absorbed (less than 7 per cent).

Absorption of drugs after 3 hours of continuous intestinal perfusion. The single perfusion technique permitted the study of the absorption of drugs which have rapid absorption rates. This technique, however, did not reveal with accuracy the considerable difference known to exist between the rates of absorption of ephedrine and of a quaternary ammonium compound because the decrease in concentration of these drugs was too small for valid comparison. Accordingly a number of the drugs that were relatively slowly absorbed by the single perfusion method were re-examined by the recirculation technique in order to magnify the differences in the concentration changes.

Definite absorption of the stronger acids, 5-nitrosalicylic and *o*-nitrobenzoic, and the stronger bases, quinine, ephedrine, tolazoline and mecamylamine, was now observed (table 5). However, no significant absorption was evident for the very strong sulfonic acid, phenol red, or for the very strong quaternary ammonium bases, Darstine (mepiperphenidol) and procaine amide ethobromide.

The possibility that the apparent rates of absorption might be due to chemical transformation was excluded in the following manner. Thirty ml. of saline were recirculated through the small intestine for 3 hours. Drugs were then dissolved in the resultant solution and incubated in a water bath at 37° C. for 3 hours. No destruction was demonstrated for *o*-nitrobenzoic acid, 5-nitrosalicylic acid, quinine, ephedrine, tolazoline or mecamylamine.

Absorption and drug binding. The possibility that the absorption of certain drugs might be affected by their binding to nondialyzable material in the intestinal perfusate was investigated by measuring the ultrafiltrability of drugs in the solution that had been recirculated for 3 hours as well as in the ileal outflow samples in the single perfusion experiments. For these experimental conditions, the binding was negligible (< 1 per

cent) for quinine, ephedrine, tolazoline, mecamylamine, Darstine, procaine amide ethobromide, Tensilon, antipyrine, aminopyrine, aniline, phenol red, *o*-nitrobenzoic acid, 5-nitrosalicylic acid, acetylsalicylic acid and salicylic acid.

Absorption in the presence and absence of bile. While bile was excluded in the majority of experiments, the absorption of all drugs listed in tables 3 and 4 except acetanilide and *p*-nitroaniline was also studied when the bile duct had not been ligated. The continuous secretion of bile, as confirmed by a detectable color in the outflowing perfusate, had no measurable effect on the rates of absorption of any of the drugs.

Isotopic exchange of water, sodium, chloride and urea. While the absorption of the isotope is not synonymous with the net absorption of the unlabeled material, the exchange rates do indicate the extent to which these small polar materials can move across the intestinal wall. The concentrations in the perfusion fluid were: deuterium oxide, 5 per cent by volume; Na^{22} and Cl^{36}, tracer amounts; and 1 *mM* of C^{14}-labeled urea per l. Absorption was studied by the single perfusion technique. Deuterium oxide was absorbed to the extent of 41 ± 6 per cent in seven animals; radiosodium, 14 ± 3 per cent and radiochloride, 8 ± 3 per cent in the same four animals; urea-C^{14}, 11 ± 2 per cent in three animals. The apparent degree of absorption of urea-C^{14} should be considered a maximal value. Urea is split to some extent by bacterial urease in the intestine permitting the absorption of some C^{14} as $C^{14}O_2$. But even if the absorption of urea were as rapid as indicated by the value of 11 per cent, it is clear that urea is absorbed substantially less rapidly than many weak organic electrolytes.

Discussion. In considering the various ways in which substances might be absorbed from the intestine, it is helpful to have in mind a general picture of cell membranes. A widely accepted concept is that cells are bounded by a lipoid membrane interspersed with small pores and that lipid-soluble substances can easily penetrate cells by dissolving in the lipid-like phase (Collander 1937; Höber, 1945). In contrast, polar substances may penetrate if they are small enough to pass through the pores. The total area of these pores is considered to be much less than that of the lipoid area. This picture of the membrane is based on experiments showing that a number of foreign compounds permeate plant cells, erythrocytes and sea-urchin eggs at rates proportional to their solubility in fat-like solvents, while certain lipid-insoluble molecules of small molecular weight appear to penetrate through pores. In addition to purely physical processes of penetration, it is recognized that specialized mechanisms are required to transport certain polar substrates across the cell boundary.

TABLE 5

Absorption of drugs after 3 hours of continuous circulation through the rat small intestine

The per cent absorbed is expressed as the mean ± the range followed by the number of experiments in parentheses.

Drug	pK_a	Per Cent Absorbed	
		Observed	*Relative to quinine*
Acid			
Phenol red	(strong)	<2 (4)	<2
o-Nitrobenzoic	2.2	11 ± 4 (3)	10
5-Nitrosalicylic	2.3	36 ± 3 (7)	38
			Relative to 5-nitrosalicylic acid
Base			
Quinine	8.4	55 ± 3 (4)	53
Ephedrine	9.6	29 ± 4 (2)	29
Tolazoline	10.3	9 ± 2 (4)	9
Mecamylamine	11.2	26 ± 2 (3)	27
Darstine	(strong)	<2 (3)	<2
Procaine amide ethobromide	(strong)	<2 (2)	<2

The question arises whether drugs are absorbed by simple diffusion or by means of specialized transport mechanisms. If a substance is absorbed by a specific transport mechanism, there should be evidence of saturation at high concentrations of substrate. The lowered proportion of glucose absorbed with increased concentration is a familiar feature of an active transport system (Höber, 1945). In contrast, the proportion of salicylic acid and aniline absorbed by the rat intestine is unchanged over a wide range of concentrations suggesting, but not proving, that these drugs are absorbed by physical processes. As further evidence of a passive process, the rates of absorption of a large number of drugs are not affected by the presence of a second drug. In contrast, glucose and galactose show mutual interference when both are present in the gut (Cori, 1926).

A clue to the nature of drug absorption is the relationship between the dissociation constant and the degree of absorption of drugs of widely

diverse chemical structures. Most drugs are weak organic acids or bases which are present in the body partly in the dissociated and partly in the undissociated form.[4] The concentration of each form depends on the dissociation constant of the organic electrolyte and on the pH of the solution in which it is dissolved. As seen in tables 3 and 4, the absorption of a large number of drugs is related to the dissociation constant. Thus most of the drugs are readily absorbed but there is a distinct reduction in the degree of absorption for strong acids with a pK_a less than 2.9 and for strong bases with a pK_a greater than 8.4. This suggests that only the unionized form of a drug readily crosses the intestinal barrier and that ions cross with difficulty. Even among highly ionized drugs, there is a distinct difference between the degree of absorption of a partially ionized compound and that of a completely ionized one (table 5).

Closer examination of the data would suggest that the simple model of a barrier selectively permeable to the unionized moiety must be qualified since there is a rapid absorption of moderately strong acids like salicylic acid. At pH 6.6, the pH of the fluid leaving the intestine, the ratio of unionized to ionized salicylic acid is about 1 to 4000. This seems too small a proportion of unionized drug to explain the rapid absorption of the compound. However this apparent inconsistency is based on the premise that the pH at the absorbing surface is also 6.6. Studies, which have been presented in preliminary form (Hogben *et al.*, 1957a), suggest that absorption may be governed by an effective pH at the epithelial boundary which is lower than 6.6.

In considering passive diffusion through pores, Höber and Höber (1937) concluded that urea, a lipid-insoluble material, was absorbed by passively penetrating through small pores in an otherwise predominantly lipoidal membrane. Our studies of the absorption of urea and D_2O suggest that molecular size may not be a major factor in determining the absorption of most drugs. Thus the polar molecule, D_2O is absorbed to the extent of only 41 per cent and the absorption of a slightly larger species, urea, is less than 11 per cent. These rates should be contrasted with rates of 50 per cent or more for the much larger lipid-soluble drugs like phenylbutazone and thiopental. Furthermore, *m*-nitrobenzoic acid is absorbed much more rapidly than *o*-nitrobenzoic acid, though both compounds have the same molecular weights; acetanilide, nitroaniline and tetraethylammonium have similar molecular weights but are absorbed at substantially different rates. It would appear that diffusion through pores may be a factor only in the absorption of compounds of very small molecular weight.

It seems reasonable to suggest accordingly that, in general, drugs of widely different chemical structures cross the intestinal barrier by diffusing through a boundary which is selectively permeable to the unionized form. This becomes comprehensible on the assumption that the boundary, like that described by Collander (1937) for cells in general, is lipoid in nature. Compounds having the same pK_a values would not necessarily penetrate a lipoid barrier at the same rates since other factors such as solubility in the membrane might be important. For example, barbital is absorbed much less rapidly than thiopental despite the similar pK_a values of the two compounds. Similarly *p*-nitroaniline is absorbed much more rapidly than antipyrine, yet both have similar pK_a values. These differences in absorption may be explained when more is known about the chemical constituents that define the physicochemical properties of the lipoid boundary.

It is noteworthy that there is an apparent upper limit to the extent of absorption, about 50 to 60 per cent, with the single perfusion technique. One of the following factors must impose this upper limit: the permeability of the intestinal mucosa, the rate of intestinal blood flow, or diffusion from the center of the stream flowing through the intestine. Since many drugs with a diversity of chemical and physical properties are absorbed at this rate, it is not likely that the permeability of the mucosa is the limiting factor under these experimental conditions. If absorption were limited by the rate of intestinal mucosal

[4] The dissociation constant of both acids and bases is expressed as a pK_a which is the negative logarithm of the acidic dissociation constant. The pK_a is defined by the Henderson-Hasselbalch equation:

$$\text{for acids} \ldots\ldots \; pK_a = pH + \log \frac{[\text{unionized acid}]}{[\text{ionized acid}]},$$

$$\text{for bases} \ldots\ldots \; pK_a = pH + \log \frac{[\text{ionized base}]}{[\text{unionized base}]}.$$

An acidic drug with a small pK_a is a very strong acid and one with a large pK_a a very weak acid. A base with a small pK_a is a very weak base.

blood flow, the binding of drugs to plasma proteins should have a substantial effect on the degree of absorption. But the plasma binding of aniline, salicylic acid, phenylbutazone, *p*-hydroxypropiophenone and thiopental ranges from 20 to 90 per cent while the compounds are all absorbed to the extent of about 60 per cent. It is possible that the upper limit of absorption of these drugs is determined by the rate at which a drug can diffuse through the intestinal solution to reach the wall. In any event, it is clear that absorption from the small intestine of the rat is a very rapid process, the half-time for many drugs being approximately five minutes.[5]

It should be made clear that the experiments we have described were designed to delineate the general characteristics of the barrier between the intestinal lumen and plasma and not to yield absolute values for the absorption of drugs as administered therapeutically. Nevertheless, it is possible to consider a number of tentative predictions. Organic electrolytes should be readily absorbed from solution if the unionized form is lipid-soluble and if the pK_a for an acid is greater than 2, and for a base is less than 11. The poor absorption of certain water-soluble drugs, formerly considered somewhat of a mystery, now becomes logical because their unionized forms are not lipid-soluble or because they are completely ionized in the gut. For example, the poor absorption of unionized substances such as mannitol and inositol (Höber and Höber, 1937) is explainable on the basis of lipid-insolubility. The absorption of quaternary ammonium compounds and sulfonic acids would be expected to be poor since they are completely ionized at physiologic hydrogen ion concentrations Levine *et al.* (1955) have suggested that the poor absorption of quaternary ammonium compounds is due to their binding to intestinal mucin, forming nonabsorbable complexes. Since we have shown that three quaternary ammonium compounds are poorly absorbed even though they are not bound to nondialyzable substances in the intestinal perfusate, it is probable that the poor absorption of quaternary ammonium compounds is due to the impermeability of the intestinal mucosa to these ions.

In actual practice, factors other than the dissociation constant and lipid solubility of a drug may limit its intestinal absorption. For example, some drugs are poorly absorbed because of their low solubility in the intestinal contents; and others because they are unstable in the gastrointestinal tract.

[5] This rapid rate of absorption is obtained in spite of a moderate distension of the gut by about eight ml. of solution and conditions that largely suspend intestinal motility. It is conceivable that under normal conditions where the intestinal contents are well mixed by the motility of the intestine, diffusion of drug to the intestinal wall would be less significant and absorption could become blood flow-limited.

SUMMARY

The relative rates of absorption of a large number of drugs have been measured by perfusing solutions of drugs through the entire small intestine of the anesthetized rat. A single perfusion of the small intestine differentiated very rapid absorption from less rapid absorption. A continuous recirculation of the drug solution through the intestine separated less rapid absorption from very slow absorption.

The degree of ionization of an organic electrolyte in solution appears to be one of the most important factors determining its absorption from the rat small intestine. Acidic drugs were rapidly absorbed if their pK_a's were greater than 3. Basic drugs were rapidly absorbed if their pK_a's were less than 8. The stronger acids and bases were relatively slowly absorbed and the absorption of very strong acids and bases was imperceptible.

Absorption of drugs from the rat small intestine can be best explained by assuming simple diffusion of unionized drug across a barrier which may be lipoidal in nature.

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[CONTRIBUTION FROM THE CHEMICAL RESEARCH LABORATORY OF FORDHAM UNIVERSITY]

SYNTHESIS OF AMINO ACIDS IN ANIMAL ORGANISMS. I. SYNTHESIS OF GLYCOCOLL AND GLUTAMINE IN THE HUMAN ORGANISM

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Received October 31, 1921

The fact that the human body is able to furnish glycocoll for the conjugation with benzoic acid has long been known and has been studied by many investigators.

Lewinski[1] observed neither benzoic acid nor benzoyl glycuronates in the urine of men to whom 12 to 20 g. of benzoic acid had been fed in small doses during the course of 12 hours. After feeding doses of 25 to 40 g., small amounts of benzoic acid (about 25% of the intake) were excreted as the free acid along with certain dextro-rotatory substances of a reducing nature indicating the presence of glycuronates. One may conclude from this that the average human adult is able to convert quantitatively about 20 g. of benzoic acid into hippuric acid. Brugsch[2] and Tsuschija,[3] on the contrary, were able to recover only about 25 to 50% of the ingested benzoic acid as hippuric acid. Dakin[4] found in his work with benzoic acid that doses of 5 to 10 g. of the acid ingested per day were almost entirely converted into hippuric acid before its elimination in the urine. The human body like that of the lower animals is therefore able to synthesize glycocoll within reasonable limits under conditions where no glycocoll occurs in the protein of the diet or even in a protein-free diet. Wiechowski[5] maintained that the glycocoll content of the tissues is insufficient to account for the quantity of glycocoll thus eliminated and that glycocoll can be synthesized at the expense of urea formed under normal conditions. Ringer[6] observed in his work an increase in the nitrogen elimination after feeding benzoic acid to animals and suggested that glycocoll resulted from the "extra destroyed protein" during this period. Epstein and Bookman,[7] experimenting on rabbits, concluded that benzoic acid as a toxic substance acts in a selective way causing the elimination of excessive amounts of nitrogen which is mostly excreted as hippuric acid nitrogen. McCollum and Hoagland[8] brought a pig of 46 kg. body weight into a condition of minimal nitrogen metabolism by feeding a diet of starch containing 75 calories for each kilogram of body weight. The diet was continued and increasing amounts of benzoic acid were fed. The results of their experiment show that when protein metabolism is reduced to a minimal level by carbohydrate ingestion the addition of benzoic acid does not affect the creatinine output, affects but little the total nitrogen, but may reduce the total elimination of urea nitrogen from 56% of the total nitrogen output to 19% of the total. This difference of 37% of the total nitrogen which is ordinarily converted into urea is under these circumstances eliminated as glycocoll.

Lewis[9] kept a man on a low protein diet containing no glycocoll for a period of 3

[1] Lewinski, *Arch. exp. Path. Pharm.*, 58, 397 (1908).
[2] Brugsch, *Z. exptl. Path. Therap.*, 5, 731 (1909).
[3] Tsuschija, *ibid.*, 5, 737 (1909).
[4] Dakin, *J. Biol. Chem.*, 8, 103 (1909).
[5] Wiechowski, *Beitr. chem. Physiol.*, 7, 201 (1906).
[6] Ringer, *J. Biol. Chem.*, 10, 327 (1911).
[7] Epstein and Bookman, *ibid.*, 10, 353 (1911).
[8] McCollum and Hoagland, *ibid.*, 16, 299 (1913-1914).
[9] Lewis, *ibid.*, 18, 225 (1914).

days. On the second day the subject ingested 8.47 g. of benzoic acid and the urine was collected at 2-hour intervals. Urea nitrogen and ammonia nitrogen were determined alone. During the first 2-hour period the amount of this nitrogen dropped from 80% to 57% of the total nitrogen; during the second 2-hour period, to 61.6%, and during the third to 64.9%, after which it returned to normal. This shows that the ingestion of 8.47 g. of benzoic acid may increase slightly the output of total nitrogen in the urine but at the same time cause a decided decrease in the amount of urea nitrogen plus ammonia nitrogen eliminated.

Thierfelder and Sherwin[10] have shown that glutamine is furnished by the human body for the detoxication of phenyl acetic acid and it was later found by Sherwin, M. Wolf and W. Wolf,[11] that 7.5 g. of glutamine was synthesized by the body on glutamine-(glutamic acid) free diet for the detoxication of ingested phenylacetic acid without materially increasing protein metabolism as shown by the total nitrogen and sulfur elimination.

In previous work a human subject had been reduced to a low protein diet for a period of only 3 days and benzoic acid fed on only 1 day. It seemed to us important that the subject be reduced to an endogenous level of nitrogen metabolism, if possible, and the experiment be extended over a longer period. We also decided to feed first benzoic acid, then phenylacetic acid and lastly a mixture of the two in order to answer if possible the following questions: (1) What is the maximum amount of urea nitrogen which the human body will "side track" for the synthesis of glycocoll, and what influence if any has this on the other nitrogen constituents of the urine? (2) Can glutamine be synthesized at the expense of the urea nitrogen alone or does the amino and amide nitrogen necessary for this compound come from different sources? (3) Can glycocoll and glutamine be formed simultaneously in the human body, and if so which one is more readily formed when a limited supply of urea nitrogen is available?

The subject, a healthy man of 80 kg. body weight, was maintained on a diet of cream, bananas, and starch or lactose for a period of 7 days.

TABLE I

INGESTED FOOD PER DAY, AND ITS ENERGY VALUE

Period	Starch	Lactose	Bananas	Cream	Total energy	Nitrogen ingested in food
Days elapsed	G.	G.	G.	G.	Calories	G.
1	180	0	50	435	2475	0.080
2	50	120	325	100	1186	0.520
3	100	120	380	30	1314	0.610
4	100	110	420	10	1278	0.670
5	40	200	545	5	1581	0.870
6	40	200	335	0	1276	0.535
7	40	150	450	0	1197	0.720

[10] Thierfelder and Sherwin, *Ber.*, **47**, 2630 (1914); *Z. physiol. Chem.*, **94**, 1 (1915).

[11] Carl P. Sherwin, M. Wolf and W. Wolf, *J. Biol. Chem.*, **37**, 113 (1918).

TABLE II—NITROGEN DATA

Period	Phenylacetic fed	Benzoic fed	Length of period	Urine Volume	Nitrogen: Total	Nitrogen: Urea	Nitrogen: Ammonia	Nitrogen: Creatinine	Nitrogen: Glycocoll	Nitrogen: Glutamine	Percentage of total nitrogen: Urea	Percentage of total nitrogen: Ammonia	Percentage of total nitrogen: Creatinine	Percentage of total nitrogen: Glycocoll	Percentage of total nitrogen: Glutamine	Percentage of total nitrogen: Rest of Nitrogen	Amt. acid detoxicated: Benzoic	Amt. acid detoxicated: Benzoic fed	Amt. acid detoxicated: Phenylacetic	Amt. acid detoxicated: Phenylacetic fed
	G.	G.	Hrs.	Cc.	G.	G.	G.	G.	G.	G.	%	%	%	%	%	%	G.	%	G.	%
1	...	...	24	2100	15.88	12.3	1.15	0.62	....	..	77	7	4	...	..	12	...	...	...	..
2	...	...	24	2020	5.91	4.66	0.52	0.58	....	..	77	9	10	...	..	4	...	...	...	..
3	...	...	24	1000	5.71	4.23	0.45	0.56	....	..	74	8	10	...	..	8	...	...	...	..
4	...	...	8	785	2.40	1.86	0.14	0.21	....	..	77	6	8	...	..	9	...	...	...	..
	...	3.0	6	685	1.95	0.93	0.18	0.15	0.32	..	48	9	8	17	..	18	2.8	93	...	..
	...	3.0	6	250	1.20	0.64	0.09	0.12	0.31	..	52	7	9	26	..	5	2.7	90	...	..
	...	...	4	53	0.54	0.35	0.05	0.05	..	..	65	10	10	...	..	15	...	...	...	..
Totals			24	1773	6.09	3.78	0.46	0.53	0.63		60	8	9	10		13	5.5	92		
5	3.3	...	6	705	1.54	0.78	0.11	0.16	....	0.30	50	7	11	...	20	12	...	...	1.5	44
	...	...	6	415	1.77	0.69	0.14	0.15	....	0.35	39	8	9	...	20	24	...	...	1.7	51
	3.3	...	6	250	1.18	0.81	0.08	0.15	....	0.11	68	7	12	...	9	4	...	...	0.5	16
	...	...	6	135	0.96	0.34	0.10	0.15	....	0.30	35	10	16	...	31	8	...	...	1.4	44
Totals			24	1505	5.45	2.62	0.43	0.61	0.31	1.06	48	8	11	...	19	14	...	...	5.1	77
6	3.3	3.0	3	820	1.07	0.20	0.09	0.10	....	0.31	19	8	10	29	29	5	2.7	89	1.5	46
	...	...	3	286	0.83	0.38	0.06	0.12	....	0.20	45	8	14	...	24	9	...	...	1.0	30
	...	...	3	222	0.66	0.46	0.05	0.11	....	0.02	70	8	17	...	3	3	...	...	0.1	3
	...	...	15	675	2.22	1.68	0.21	0.27	....	....	76	9	12	...	..	3	...	...	...	..
Totals			24	2003	4.78	2.72	0.41	0.60	0.31	0.53	57	8	13	6	11	5	2.7	89	2.6	79
7	4.0	...	4	750	1.00	0.44	0.06	0.12	....	0.32	44	6	12	...	30	8	...	...	1.5	39
	...	...	4	555	1.31	0.32	0.14	0.11	....	0.50	24	11	8	...	38	18	...	...	2.4	60
	6.0	...	4	520	1.01	0.29	0.10	0.10	....	0.38	29	10	10	...	37	14	...	...	1.8	30
	...	...	4	255	0.97	0.12	0.10	0.09	....	0.51	12	10	9	..	52	16	...	...	2.5	41
			[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]			[illegible]	[illegible]
Totals			[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]			[illegible]	[illegible]

preceding table shows the weight of each substance eaten per day as well as its calorific value.

This diet which is practically nitrogen free insofar as utilizable nitrogen is concerned[12] is also of sufficient calorific value for the needs of a man undergoing only a small amount of physical exertion. Agar-agar was ingested in sufficient amounts to cause the regular evacuation of the intestine and thus avoid the absorption of nitrogenous end products of putrefaction.

In Table II, the days of acid feeding have been divided into sub-periods of varying length in order to study the amount of different substances excreted immediately after the ingestion of a given amount of acid. The [illegible] ingested during a certain sub-period of the day was always taken at the beginning of that sub-period, and the entire output of urine collected during each sub-period was treated as a single unit. Also for each one of the last 4 days a sum total is given of all the sub-periods that the entire 24 hour period may be compared to one of the first 3 days of the control period.

A brief survey of Table II brings out clearly several interesting facts. Neither benzoic acid nor phenylacetic acid when fed in moderate doses (3 to 10 g.) to man acts as a stimulator of endogenous metabolism when the subject has been reduced practically to a state of endogenous protein metabolism. This is shown by the fact that the amount of total nitrogen excreted is not increased during the period of acid ingestion. It is also seen that neither glycocoll nor glutamine is built at the expense of "extra dietary protein" but rather at the expense of one or more of the nitrogenous constituents of the urine, and of these only urea is affected to [illegible]ed degree.

Urea, during the control period, maintained an average of 74 to 77% of the total nitrogen, while during the feeding period it dropped to 60%, 57% then to 48% and finally to 28% of the total nitrogen of the entire [illegible] period. If taken by sub-periods even more striking evidence is [illegible] the inroads made on urea nitrogen for the purpose of glycocoll [illegible]glutamine synthesis. After a dose of 3 g. of benzoic acid on the fourth day [illegible]wed at the end of 6 hours by a second dose of 3 g. the urea nitrogen dropped to 48% and 52% respectively of the total nitrogen. On the fifth day 2 doses each of 3.3 g. of phenylacetic acid caused a further drop of the urea nitrogen to 39% and 35% respectively of the total nitrogen. It is interesting here to note that the effect of the benzoic acid on urea nitrogen was immediate and largely confined to the sub-period at the beginning of which it was fed, while the greatest effect of the phenylacetic acid was seen in a later sub-period. One may infer from this either the benzoic acid is more quickly absorbed than the phenyl-

[12] [illegible]k, "Science of Nutrition," W. B. Saunder and Co., 1919, p. 355.

acetic acid, or that glycocoll is more easily synthesized by the body t is glutamine. This latter inference one may explain on the ground t glutamine not only demands 2 atoms of nitrogen for its synthesis but be sides an amine requires also an amide group.

During the seventh day of the experiment the greatest reduction is fo in the urea nitrogen. At the beginning of the first 4-hour sub-period 4 g. of phenylacetic acid was ingested. This reduced the urea nitrogen to 44% of the total nitrogen, but caused a still greater fall in urea nitrogen (24% of total nitrogen) during the subsequent 4-hour sub-period. At this time a second dose of 6 g. of phenylacetic acid was ingested by subject, but the percentage of urea nitrogen rose to 29%, only to fa hours later to the very low value of 12% of the total nitrogen. This value was considerably lower than had been previously obtained. McCollum found that the urea nitrogen fell to only about 20% of the total nitrogen after the feeding of large doses of benzoic acid to a pig, and that larger doses of the acid caused only an increase in protein metabolism and a sub sequent increase in total nitrogen excreted. In this case, however, t entire 24 hours was considered as a unit. On the seventh day of our experiment it will be seen that the urea nitrogen for the entire 24 hours formed 28% of the total nitrogen.

The ammonia nitrogen was little, if at all, affected by the feeding of either of these organic acids. It has been previously shown by McCollum that an organic acid is detoxicated at the expense of the urea nitrogen and an inorganic acid (hydrochloric acid) at the expense of the ammonia nitrogen.

It seemed probable that the amino nitrogen for glutamine synth would be taken from the urea fraction, but just possible that the a monia nitrogen might be required to build the amide portion of the m cule. That this is not the case is shown by the fact that there is no crease in ammonia nitrogen after the ingestion of 10 g. of phenylac acid on the last day of the experiment.

Creatinine was unaffected by the acid ingestion, for the creatin value remained particularly constant during the last days of the exper ment.

Lewinski[1] found that 10 to 20 g. of benzoic acid ingested by a man quantitatively converted into hippuric acid may remove as much as 3 of the total nitrogen in the form of glycocoll (hippuric acid) nitrogen Ringer[2] obtained much the same results with goats. Wiechowski,[3] employing guinea pigs, found that glycocoll nitrogen might run as high as 64% after the feeding of benzoic acid. After feeding the 6 g. of benzoic acid on the fourth day of our experiment 10% of the total nitrogen was found in the form of hippuric acid nitrogen. On the fifth day 19% of the total nitrogen appeared in the form of glutamine nitrogen. On

sixth day after feeding 3.3 g. of phenylacetic together with 3 g. of benzoic acid 6% of the total nitrogen appeared as glycocoll nitrogen and 11% as glutamine nitrogen. On the seventh day 35% of the total nitrogen was excreted in the form of glutamine nitrogen, which is comparable to the values found by some of the other investigators. Only in certain sub-periods of certain days does the excretion of glycocoll and glutamine nitrogen reach the value quoted by Wiechowski. In the first sub-period (3 hours) of the sixth day after the ingestion of 3.3 g. of phenylacetic acid and 3 g. of benzoic acid, 29% of the total nitrogen was found in the form of glycocoll nitrogen and 29% in the form of glutamine nitrogen, a total of 58%. Again in the fourth sub-period (4 hours) of the seventh day after the ingestion of 10 g. of phenylacetic acid 52% of the entire nitrogen output of the sub-period appeared in the urine as glutamine nitrogen.

Moderate doses of benzoic acid are probably excreted quantitatively in urine as hippuric acid. We found 89% to 93% so excreted within 6 hours after a 3g. dose. Phenylacetic acid seems to require about twice as much time as benzoic acid for its excretion (in the form of phenylacetyl glutamine). Its detoxication, moreover is apparently not so complete, for though after a dose of 10 g. we were able to recover 95% of it from the 24-hour sample of urine, still in other cases after small doses we recovered only about 78% of the amount fed.

Apparently the body builds both glutamine and glycocoll simultaneously as readily and efficiently as either of the compounds alone. After the ingestion of 3 g. of benzoic acid 92% of it was converted into hippuric acid within 6 hours. After the ingestion of 3.3 g. of phenylacetic acid on another day 44% of it was converted into phenylacetyl glutamine within 6 hours. When 3 g. of benzoic acid and 3.3 g. of phenylacetic acid were ingested simultaneously 89% of the benzoic acid and 46% of the phenylacetic acid were changed into their respective detoxication products within a period of 6 hours.

Summary

It has been shown that man will synthesize glycocoll at the expense of urea as do the lower animals. The synthesis of another amino acid (glutamine) at the expense of urea nitrogen has also been demonstrated in the case of a man. The two amino acids may be built simultaneously as readily as either compound alone.

During the period in which these amino acids were being synthesized in the organism, urea nitrogen dropped from about 75% of the total nitrogen to 28%, and during a sub-period of a certain day, to the extremely low value of 12% of the total nitrogen.

After feeding a moderate dose of benzoic acid (3.3 g.), glycocoll for its

detoxication is built within 6 hours, while for the detoxication of a corresponding dose of phenylacetic acid a somewhat longer period of time is required for the synthesis of glutamine.

NEW YORK, N. Y.

Fd Cosmet. Toxicol. Vol. 8, pp. 369–380. Pergamon Press 1970. Printed in Great Britain

Toxicological Evaluation of some Combinations of Food Preservatives

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(*Received 12 August 1969*)

Abstract—Short- and long-term studies are reported in which preservatives were administered orally, singly or in pairs, to rats and mice. Daily doses of nisin (0·4 mg/kg), sorbic acid (40 or 80 mg/kg), benzoic acid (40 or 80 mg/kg) and sodium bisulphite (80 or 160 mg/kg), and mixtures of benzoic acid/sodium bisulphite (40/80 or 80/160 mg/kg) and sorbic acid/nisin (40/2 or 80/4 mg/kg) were given to mice or rats for 3, 11 or 18 months, and in a further test nisin (0·4–400 mg/kg/day), sorbic acid (40 mg/kg/day) and a polymeric impurity derived from the sorbic acid were tested in mice for 2 months. In addition to the effects of these substances on growth and survival, haematology and blood chemistry (in rats), kidney function (in rats) and reproduction (in mice), the effects of certain stress factors, including dietary restriction, low temperature and carbon tetrachloride administration, on the potential toxicity of the preservatives were studied in both species.

In general, these doses showed some adverse effects in both rats and mice, particularly on growth, survival and susceptibility to stress, but sorbic acid appeared to be the least toxic. The effects of the benzoic acid/sodium bisulphite mixture were generally greater than those of the compounds given singly, while the reverse was the case with the sorbic acid/nisin mixture. The antibiotic, nisin, increased the growth rate when given alone. The authors' recommendation that the use of benzoate and sulphite as preservatives should be further restricted and that sorbic acid should be used instead where possible is being followed in the USSR.

INTRODUCTION

Despite the development of newer techniques, chemical preservatives still retain their economic importance for extending the shelf-life of foods and conserving their nutritional value. Until recently sulphites and benzoates found fairly wide use in the USSR as food preservatives, but now their use is restricted as much as possible. Limited amounts of sulphurous acid are permitted in certain foods, including some wines, jams, fruit candies, starch, tomato puree, dried fruits, fruit juices and some fresh and semi-processed fruits. Benzoic acid is also used, for preserving gelatin, fruit candy and waste, pastilles, margarine, egg mayonnaise, salmon caviare, spiced sprats and some berries and fruit products, and sodium benzoate is permitted in fruit juices, fish preserves and margarine.

Recently sorbic acid and the antibiotic, nisin, have been proposed for extensive use as preservatives, but their safety-in-use had to be established before they could be added to the existing list of permitted food additives. It was decided, therefore, that the technological and economic questions concerning these materials should be studied at the All Union Institute of the Food Canning Industry, while we undertook the toxicological studies.

Toxicological data are available for the administration of each of these compounds individually to experimental animals (Joint FAO/WHO Expert Committee on Food Additives, 1962), but not for their administration in combination. According to the literature, sodium bisulphite fed at dietary levels of 0·05% for up to 2 yr had no adverse effects but levels of 0·1% or higher reduced weight gain and induced histopathological changes in some organs (Fitzhugh, Knudsen & Nelson, 1946). The authors attributed these effects to avitaminosis B_1 following destruction of this vitamin in the diet by the sodium bisulphite. Intubated doses of up to 2·5 g sodium bisulphite given daily to dogs for up to 419 days had no toxic effects. Sodium benzoate had no adverse effects in rats given dietary levels of up to 4% for 90 days, but levels of 8% increased mortality, reduced weight gain and caused hypertrophy of the liver and kidneys (Deuel, Alfin-Slater, Weil & Smyth, 1954). No toxic effects occurred in four generations of rats fed 0·5 or 1·0% in the diet (Kieckebusch & Lang, 1960). High levels of protein in the rat diet have a protective action against this preservative (Lang, 1961). Sorbic acid had no toxic effects when fed in the diet of rats at levels of up to 8% for 90 or 1000 days (Deuel *et al.* 1954; Lang, 1960). No toxic effects were detected in rats fed 0·1–0·3% nisin in the diet, with cheese, for periods of 12 wk–1 yr, survival, weight gain, reproduction indices, haematology, gastro-intestinal and kidney function and organ histology being within the limits of the control animals (Hawley, 1958; Frazer, 1960).

Since mixtures of food additives are ingested in a normal diet, it was considered appropriate to carry out short- and long-term oral toxicity tests in rats and mice on combinations of benzoic acid and sodium bisulphite and of sorbic acid and nisin, as well as on the individual compounds.

EXPERIMENTAL

Materials. Benzoic acid and sodium bisulphite were analytical-grade reagents obtained commercially. Sorbic acid, obtained commercially, contained an unknown polymeric impurity (0·02–0·07% ashed residue) which was insoluble in water, sparingly soluble in acids or alkali and moderately soluble in chloroform. Nisin with an activity of 1×10^6 IU/g was purchased from Aplin & Barrett Ltd., Yeovil, Somerset, UK. It was of largely unknown composition but contained a stabilizer.

Animals. Both sexes of cross-bred white mice and Wistar rats were used, the initial body weights varying according to the nature of the experiment. Mice were housed in groups of 10–25 and rats in groups of five.

Experimental design and conduct

2-Month study in mice. Groups of 25 male and 25 female mice (initial body weight 8–10 g) received for 2 months by oral intubation daily doses of 0·4, 4·0 or 400 mg nisin/kg/day (approximately equivalent to 0·002, 0·02 and 2·0% in human food). Groups of 25 male and 25 female mice (initial body weight 15–20 g) received the same doses of nisin for a similar period. Sorbic acid (40 mg/kg/day) and the polymeric impurity obtained from it (0·8 or 800 mg/kg/day) were given to similar groups of male and female mice from each body-weight range. The mice were observed for general condition and behaviour, and survival, food consumption and weight gain were recorded daily. Tests were carried out to determine the effects of hunger, physical stress and poisoning with carbon tetrachloride on the test animals, compared with untreated controls.

3-Month study in mice. Groups of 50 male and 50 female mice (initial body weight 8–10 g)

received, by oral intubation, sorbic acid or benzoic acid in doses of 80 mg/kg/day), sodium bisulphite (160 mg/kg/day) or nisin (4 mg/kg/day). Similar groups received mixtures of the same levels of benzoic acid and sodium bisulphite and of sorbic acid and nisin. The observations and tests performed on these mice were the same as those described for the 2-month study, except that a co-carcinogenicity test with Ehrlich ascites carcinoma was added.

17-Month study in mice. Groups of 25 male and 25 female mice (initial body weight 10–15 g) and groups of 25 male and 25 female mice (initial body weight 16–20 g) were given sorbic acid or benzoic acid in doses of 40 mg/kg/day, sodium bisulphite (80 mg/kg/day) or nisin (2 mg/kg/day). Similar groups received mixtures of the same levels of benzoic acid and sodium bisulphite and of sorbic acid and nisin. The test compounds were fed in a paste prior to the main feed, feed and water were provided *ad lib.* and a control group received only the basic diet. The parameters studied on the animals in this test were those listed for the 2-month study, except that poisoning by carbon tetrachloride was not studied and in addition organ weights were recorded. After 8 months on the preservative-combination or control diets some animals were mated and reproduction was studied over five generations. The weight gain of the litters after weaning (at 8–10 g body weight) was recorded for 3·5 months.

18-Month study in rats. Groups of ten male and ten female rats (initial body weight 100–120 g) received sorbic acid or benzoic acid (40 mg/kg/day), sodium bisulphite (80 mg/kg/day) or nisin (2 mg/kg/day). Groups of 50 male and 50 female rats (initial body weight 100–120 g) received sorbic acid (40 mg/kg/day) in combination with nisin (2 mg/kg/day), or benzoic acid (40 mg/kg/day) in combination with sodium bisulphite (80 mg/kg/day). Test compounds were fed in a paste prior to the main feed, while control groups received the basic diet alone. Food and water were provided *ad lib.* Food and water consumption, weight gain and the effects of stress factors were recorded. Blood morphology was studied and the following were estimated: titre of serum complement by our micro-modification of the classical method; phagocytic activity of leucocytes by the slide method of Rost and Stenco as described by Predtechenskii (1959); serum-cholesterol level by Ravin's method (Petrun'kina, 1959); blood alkalinity (Panisyuk, 1962); blood hexoses by Natelson's method (Todorov, 1960); blood morphology; erythrocyte sedimentation rate by a modification of Efmann's method (Mukherina, 1962); C-reactive protein in serum (Tashinin, 1962).

Effect of stress factors on preservative toxicity in mice and rats

Food restriction. At the termination of the feeding studies, surviving mice were subjected to a restricted dietary intake for up to 5 days, the degree of restriction being 50% after the 2-month, 90% after the 3-month and 100% after the 17-month test. Rats (ten/group) were starved for up to 13 days. Both rats and mice received water *ad lib.* and administration of the preservatives was continued at the same dose levels by oral intubation. Weight loss, mortality rate and, in the case of the rats, the rate of weight recovery after the return of survivors to a normal diet, were recorded.

Swimming test. All mice surviving at the end of the 2- and 17-month studies were fitted with a 2-g weight on the tail and left to swim in a water bath at 15–18°C. The time until they sank exhausted was recorded.

Low temperature. Groups of 15 rats from those fed combinations of preservatives were exposed to cold (−2–+8°C) for 12–24 hr at the end of the 18-month study.

Centrifugation. To study the functional efficiency of the rats' circulatory-respiratory system

after the prolonged consumption of mixtures of preservatives, groups of 15 rats were subjected to a rotation rate of 90–300 rev/min for 15–30 min.

Carbon tetrachloride detoxification test. This test was carried out to determine the effect of feeding preservatives on the detoxifying action of the liver. Groups of 7–32 mice that had received single preservatives for 2 or 3 months were given carbon tetrachloride by oral intubation in a single dose of 0·1 ml/mouse in sunflower oil (1:1 solution). After receiving one of the combinations of preservatives for 18 months groups of 9–15 rats were treated with orally-intubated carbon tetrachloride either in a single dose of 0·6 ml/rat or in daily doses of 0·2–0·3 ml/rat for 15 days. The mortality rate in these animals was compared with those from the feeding-test control groups given similar doses of solvent.

Kidney function test. Groups of 15 rats that had been given combinations of preservatives were given a kidney function test at the end of the 18-month period of feeding. A saturated solution of K_2HPO_4 was administered in doses of 4 ml/rat twice daily for 6 wk, and haematological studies and urinary protein determinations were carried out.

RESULTS

2-Month study in mice

Weight gain after 15, 30 and 60 days on test showed little divergence from the controls in any group, except in males receiving 0·4, 4·0 or 400 mg nisin/kg/day, in which the weight gain was significantly increased in the group with the lower initial body weight, and in males and females of the group with the higher initial body weight given the polymeric impurity from sorbic acid in a dose of 800 mg/kg. Survival and food consumption in the test groups did not differ significantly from those of the controls. The effect of food restriction on body weight and survival is shown in Table 1. The weight loss was highest in groups receiving the higher doses of nisin and the lower level of polymeric impurity, but survival time was highest in the two groups given the impurity. The same tendency was found in the effects of physical stress and carbon tetrachloride poisoning (Table 2), with mice given nisin being more susceptible than the animals of the other groups, and those given sorbic acid or the lower level of sorbic acid impurity showing a lower degree of mortality than the controls.

3-Month study in mice

Highest mortality rates at 2·5 months were recorded in mice given nisin or the benzoic acid/sodium bisulphite combination (Table 3). Animals given benzoic acid or sodium bisulphite separately showed about twice as high a survival rate as was seen with the combination of the two.

With the exception of males of the nisin group, test animals did not grow as well as the controls at 3 months, but this finding was not associated with a reduced feed intake (Table 4). The weight gain of mice fed the benzoic acid/sodium bisulphite combination was higher than that of mice fed either of the components, whereas in the case of nisin and sorbic acid the reverse was the case.

Under conditions of restricted feed intake mice on benzoic acid, the benzoic acid/sodium bisulphite combination or nisin showed the highest mortality rate (Table 1). The effect of carbon tetrachloride on the mortality of mice fed the preservatives for 3 months can be seen from Table 2. Mice given benzoic acid or sodium bisulphite showed a higher mortality rate than controls but those given sorbic acid were not susceptible to the dose of carbon tetrachloride given. The results of the co-carcinogenicity test are reported below.

Table 2. *Effect of 0·1 ml carbon tetrachloride on the mortality of preservative-treated mice*

Test compound	Daily intake (mg/kg)	Mortality (%)
None (control)*	—	28·6
Benzoic acid*	80	62·5
Sodium bisulphite*	160	45·0
Sorbic acid*	80	0·0
Control†	—	22·3
Nisin†	0·4	46·7
	4·0	56·4
	400·0	70·0
Sorbic acid†	40·0	14·8
Sorbic acid impurity†	0·8	0·0
	800·0	26·6

* Groups of ten mice fed the control or preservative diet for 3 months.
† Groups of 7–20 mice fed the control or preservative diet for 2 months.
The effects of carbon tetrachloride were not tested on mice fed the combinations of preservatives.

Table 3. *Survival rate at 2·5 and 8 months in mice given preservatives orally, alone or in combination*

Test compound(s)	Daily intake (mg/kg)	Survival (%) in Males at month 2·5*	Males at month 8†	Males at month 8‡	Females at month 2·5*	Females at month 8†	Females at month 8†
None (control)	—	60	72·5	78·5	62	—	—
Benzoic acid	80	68			68		
Sodium bisulphite	160	70			68		
Sorbic acid	80	98			52		
Nisin	4	12			32		
Benzoic acid/sodium bisulphite	80/160	30			38		
	40/80		44·4	35·4		28·5	55·3
Sorbic acid/nisin	80/4	48			74		
	40/2		22·4	9·4		37·9	73·0

* Groups initially comprised 50 male or 50 female mice, weighing 8–10 g in either case.
† Groups initially comprised 5–20 mice of both sexes, weighing 10–15 g. Controls were males only.
‡ Groups initially comprised 20–50 mice of both sexes, weighing 16–25 g. Controls were males only.

17-Month study in mice

Growth and survival. The survival rate of these mice at 8 months can be seen in Table 3. Animals in the control groups had a better survival rate than those receiving the preservative

combinations. The poorest survival was in males receiving sorbic acid in combination with nisin, but this group registered the greatest weight gain as shown in the reproduction study data (Table 5). This was especially marked in the group with initial body weights between 10 and 15 g. The weights of liver, kidney and testes relative to body weight in mice sacrificed at the end of the test period were lower in the group given sorbic acid alone than in all the other groups.

Reproduction study. In the four generations of offspring, the weight gain of the litters was studied for 3-5 months after weaning (at 8–10 g body weight) and the effects of the preservative combinations on the weight of the animals at 3·5 months are shown in Table 5. Those fed sorbic acid in combination with nisin grew better on the whole than mice given benzoic acid in combination with sodium bisulphite.

Effects of stress. In the swimming test a better time was recorded for the groups of mice given sorbic acid alone or in combination with nisin than for mice given benzoic acid alone or in combination with sodium bisulphite. The effect of feed restriction on body weight and survival can be seen in Table 1. Percentage mortality after 5 days of feed restriction was highest in groups receiving benzoic acid alone or in combination with sodium bisulphite. The time required to regain lost weight was also slightly greater in these groups.

Tumour incidence. After the feeding of preservative diets for 17 months, 8/100 mice in the benzoic acid/sodium bisulphite group of the first generation and 1/8 in the third generation of the same group were found to have malignant tumours. No tumours were found in the control or sorbic acid/nisin groups. In connection with these findings we decided to study the possible co-carcinogenicity of the preservatives using the tumour transplantation test. Ehlich ascites mouse carcinoma was transplanted intraperitoneally into mice which had been fed for 3-months on the test diets. The tumour growth was greatest in mice that had received sodium bisulphite, and was less marked in mice given bisulphite in combination with benzoic acid or benzoic acid alone. The tumour growth in mice given sorbic acid alone or in combination with nisin was comparable with that in the control group.

Long-term effects in rats

Rats fed the sorbic acid/nisin mixture grew better than those fed benzoic acid/sodium bisulphite over an 18 month period (Table 6). The titre of serum complement and the phagocytic activity of the leucocytes were also higher in the former than in the latter group, but the level of serum ceruloplasmin was higher in the latter than in the former. Cold stress, centrifugation and kidney loading with Na_2HPO_4 had most effect in rats given the benzoic acid/sodium bisulphite combination.

Blood alkalinity, C-reactive protein levels and blood morphology were comparable in all groups including the controls. But the erythrocyte sedimentation rate was increased in rats fed the benzoic acid/sodium bisulphite combination, in which group the level of blood ketones was 1·97 mg/100 ml, compared with 2·5 mg/100 ml in the control group and 3·4 mg/100 ml in the sorbic acid/nisin group.

The results in Table 7 indicate that rats fed benzoic acid for 18 months developed some tolerance to a lethal dose of the compound given terminally. Rats on a sorbic acid/nisin diet tolerated carbon tetrachloride better than did those on the sodium bisulphite/benzoic acid diet. In the latter group [illegible] of the animals showed signs of haemorrhagic keratoconjunctivitis, which developed 5 months after the beginning of the study. The effects of starvation (with water available ad lib.) were intermediate, rats in all the test groups surviving longer than the controls.

Table 4. *Mean values of weight gain and daily intake of feed and water in mice given preservatives orally, singly or in combination for 3 months*

Test compound(s)	Daily intake (mg/kg)	Weight gain (% of initial body weight)		Daily feed intake (g)		Daily water intake (ml)	
		Males	Females	Males	Females	Males	Females
None (control)	—	199	167	2·33	2·81	3·67	4·14
Benzoic acid	80	125	118	2·57	2·65	[illegible]	[illegible]
Sodium bisulphite	160	143	134	2·43	1·98	[illegible]	[illegible]
Sorbic acid	80	143	144	2·17	2·13	[illegible]	[illegible]
Nisin	4	[illegible]	143	3·36	4·31	[illegible]	[illegible]
Benzoic acid/sodium bisulphite	80/160	174	141	[illegible]	[illegible]	[illegible]	[illegible]
Sorbic acid/nisin	80/4	[illegible]	145	[illegible]	[illegible]	[illegible]	[illegible]

Groups of 50 males and 50 females (initial body weight 8–10 g) were used.
Feed and water consumption were recorded daily throughout the test period.

Table 5. *Weight gain at 3·5 months in four generations of mice given combinations of preservatives from weaning to mating*

Test compounds	Dosage level (mg/kg)	Weight gain (% weight at weaning) at 3·5 months for generation							
		F_1		F_2		F_3		F_4	
		Males	Females	Males	Females	Males	Females	Males	Females
None (control)	—	218	200	147	118	228	212	105	80·5
Benzoic acid/sodium bisulphite	40/80	150	145	103	99	180	217	171	212
Sorbic acid/nisin	40/2	257	202	138	118	219	199	242	187

Table 6. *Mean values of weight gain and daily intake of feed and water in rats fed various preservatives singly or in combination for 18 months*

Test compound(s)	Daily intake (mg/kg)	Weight gain at 18 months (% of initial body weight)		Daily feed intake (g)		Daily water intake (ml)	
		Males	Females	Males	Females	Males	Females
None (control)		203·49	168·8	17·2	9·6	37·8	26·4
Benzoic acid	40			14·8	13·1	28·6	26·3
Sodium sulphite	80	-		14·5	9·8	32·0	25·7
Sorbic acid	40			13·6	9·7	38·7	27·3
Nisin	2			16·8	9·2	39·5	32·6
Benzoic acid/sodium bisulphate	40/80	174·8	151·8				
Sorbic acid/nisin	40/2	253·7	203·0				

Weight gain was estimated in groups of 50 rats (initial body weight 100–120 g) and feed and water intakes in groups of ten.

Table 6. *Mean values of weight gain and daily intake of feed and water in rats fed various preservatives singly or in combination for 18 months*

Test compound(s)	Daily intake (mg/kg)	Weight gain at 18 months (% of initial body weight)		Daily feed intake (g)		Daily water intake (ml)	
		Males	Females	Males	Females	Males	Females
None (control)		203·49	168·8	17·2	9·6	37·8	26·4
Benzoic acid	40			14·8	13·1	28·6	26·3
Sodium metabisulphite	80	-		14·5	9·8	32·0	25·7
Sorbic acid	40			13·6	9·7	38·7	27·3
Nisin	2			16·8	9·2	39·5	32·6
Benzoic acid/sodium bisulphate	40/80	174·8	151·8				
Sorbic acid/nisin	40/2	255·7	203·0				

Weight gain was estimated in groups of 50 rats (initial body weight 100–120 g) and feed and water intakes in groups of ten.

Table 7. *Effect of carbon tetrachloride and sodium bisulphite and benzoate on mortality in rats fed preservatives singly or in combination for 18 months*

Test compound(s) fed for 18 months	Secondary compound and dose		Mortality (%)
None (control)	Sodium benzoate (mg/kg)	3600	100
Sorbic acid/nisin		3400	71·5
Sodium bisulphite/benzoic acid		3400	100
Benzoic acid		4000	25
None (control)	Sodium bisulphite (mg/kg)	860	100
		430	100
Sorbic acid/nisin		860	66·6
		430	33·3
Sodium bisulphite/benzoic acid		860	100
		430	60
Sorbic acid/nisin	Carbon tetrachloride (ml)	0·6	33·3
		0·2	11·0
Sodium bisulphite/benzoic acid		0·6	60·0
		0·2	27·0

Groups of 3–15 rats were used.

DISCUSSION

The data presented suggest that the preservatives tested had some slight adverse effects on rats or mice in a dosage range equivalent to the maximum levels at which these preservatives may occur in the human diet. The toxic effects of benzoic acid and sodium bisulphite when administered in combination appeared to be synergistic or additive to some extent, while there were indications that the reverse was the case with the sorbic acid/nisin combination. This is an important consideration in the control of the use of preservatives for extending the shelf-life of food for human consumption. In our experiments the sorbic acid showed the lowest toxicity and changes that were recorded in some tests were not considered to be pathological. Moreover, this unsaturated fatty acid has some beneficial effect on the animal organism, being not only a good preservative for food but a nutritional additive with some features of the vitamins. Therefore it was considered reasonable to recommend to the Food Hygiene Department of the Ministry of Health of the USSR a maximum restriction on the permitted list of foods preserved with sodium bisulphite (and other compounds of sulphurous acid) and with benzoates. This recommendation has been accepted.

As the data obtained for nisin were not convincing, it was decided to carry out a further study with nisin manufactured in the Soviet Union. This was recently completed (Shillinger, Bogoroditskaya & Osipova, 1969) and the home-produced nisin was found to be less toxic than the imported. However nisin is an antibiotic, a fact confirmed by our own studies (particularly the finding of weight stimulation in the test animals) and its use as a food preservative should therefore be limited. The high content of nisin occurring naturally in some dairy products is not a sound reason for unrestricted use of this compound as a food preservative, but there is some case for using nisin in combination with sorbic acid, as we have recommended for some products. In the present situation it is considered reasonable to restrict the use of benzoates and sulphites at the expense of extending the use of sorbates, a trend which is at present being followed in the USSR.

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Evaluation toxicologique de certaines combinaisons de produits de conservation des aliments

Résumé—L'article décrit des études à court et à long terme, au cours desquelles des produits de conservation ont été administrés par voie orale, isolément ou par paires, à des rats et à des souris. Des rats ou des souris ont reçu pendant 3, 17 ou 18 mois des doses quotidiennes de nisine (2 ou 4 mg/kg), d'acide sorbique (40 ou 80 mg/kg), d'acide benzoïque (40 ou 80 mg/kg) ou de bisulfite de sodium (80 ou 160 mg/kg) ou de mélanges d'acide benzoïque et de bisulfite de sodium (40/80 ou 80/160 mg/kg) et de mélanges d'acide sorbique et de nisine (40/2 ou 80/4 mg/kg). La nisine (0,4–400 mg/kg/jour), l'acide sorbique (40 mg/kg/jour) et une impureté polymère dérivée de l'acide sorbique ont été testés pendant 2 mois sur des souris au cours d'un autre essai. En plus des effets de ces substances sur la croissance et sur la survie, sur l'hématologie et la chimie du sang (chez les rats), sur la fonction rénale (chez les rats) et sur la reproduction (chez les souris), on a étudié, chez les deux espèces animales, les effets de certains facteurs de stress: restriction alimentaire, basse température et administration de tétrachlorure de carbone, sur la toxicité potentielle des produits.

D'une manière générale, les doses précitées ont eu certains effets nocifs, autant chez les rats que chez les souris, plus particulièrement sur la croissance, la survie et la sensibilité au stress. L'acide sorbique s'est révélé le moins toxique. Les effets du mélange d'acide benzoïque et de bisulfite de sodium étaient généralement plus prononcés que ceux de ces produits administrés séparément, mais l'inverse a été observé pour le mélange d'acide sorbique et de nisine. Administré seul, l'antibiotique qu'est la nisine accélérait la croissance chez les rats. On recommande de restreindre davantage l'emploi du benzoate et du sulfite comme produits de conservation des aliments et de les remplacer autant que possible par l'acide sorbique. Cette recommendation est déjà suivie en URSS.

Toxikologische Prüfung einiger Kombinationen von Konservierungsmitteln für Nahrungsmittel

Zusammenfassung—Es wird über kurz- und langzeitige Untersuchungen berichtet, bei denen Konservierungsmittel einzeln oder paarweise Ratten und Mäusen oral verabreicht wurden. Tägliche Dosen von Nisin (2 oder 4 mg/kg), Sorbinsäure (40 oder 80 mg/kg), Benzoesäure (40 oder 80 mg/kg) und Natriumbisulfit (80 oder 160 mg/kg) und Mischungen von Benzosäure/Natriumbisulfit (40/80 oder 80/160 mg/kg) und Sorbinsäure/Nisin (40/2 oder 80/4 mg/kg) wurden 3, 17 oder 18 Monate Mäusen oder Ratten verabreicht, und bei einem weiteren Versuch wurden 2 Monate lang Nisin (0,4–400 mg/kg/Tag), Sorbinsäure (40 mg/kg/Tag) und eine aus der Sorbinsäure isolierte polymere Verunreinigung an Mäuse verabreicht. Ausser den Wirkungen dieser Substanzen auf das Wachstum und das Überleben, die Hämatologie und Blutchemie (bei Ratten), die Nierenfunktion (bei Ratten) und die Fortpflanzung (bei Mäusen) wurden die Wirkungen bestimmter Stressfaktoren einschliesslich der Futtereinschränkung, der Einwirkung niedriger Temperaturen und der Anwendung von Tetrakohlenstoff auf die mögliche Toxizität der Konservierungsmittel bei beiden Spezies untersucht.

Im allgemeinen zeigten diese Dosen einige nachteilige Wirkungen bei Ratten und Mäusen, besonders auf das Wachstum, das Überleben und die Stressempfindlichkeit, jedoch schien die Sorbinsäure am wenigsten toxisch zu sein. Die Wirkungen der Benzoesäure/Natriumbisulfit-Mischung war im allgemeinen grösser als die der einzeln verabreichten Verbindungen, während es im Fall der Sorbinsäure/Nisin-Mischung gerade umgekehrt war. Das Antibioticum Nisin erhöhte die Wachstumsgeschwindigkeit bei Einzelverabreichung. Der Autor empfiehlt, die Verwendung von Benzoat und Sulfit als Konservierungsmittel weiter einzuschränken. Stattdessen sollte wie in der UdSSR Sorbinsäure stets verwendet werden, wenn es möglich ist.

Vol. 67 (2): 287-291, 1957. Biochem. J.

The Formation of Glycine and Serine

THE INFLUENCE OF THE ADMINISTRATION OF GLYCINE, DL-SERINE AND OTHER COMPOUNDS ON LEVELS OF TISSUE GLYCINE AND SERINE

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(*Received* 8 *March* 1957)

The rates of conversion of glycine into serine and serine into glycine have been determined by administration of isotopically labelled glycine and serine (Arnstein & Neuberger, 1953; Elwyn & Sprinson, 1954; Arnstein & Keglović, 1956; Arnstein & Stanković, 1956). The present investigation, which is concerned with the net synthesis of glycine from serine, and of serine from glycine, was carried out with unlabelled glycine and serine and the methods were similar to those used in a previous investigation of tissue-glycine levels (Simkin & White, 1957). Greenberg & Sassenrath (1955) have made a more extensive but less detailed investigation of the effect of administration of several amino acids including glycine and serine.

METHODS

Animals, diet and method of dosage were described by Simkin & White (1957). The dose level of amino acids and of sodium benzoate was usually about 400 μmoles/100 g. body wt. In the rat the volume injected was generally 1·0 ml., except with DL-serine where its low solubility made it necessary to use 1·5–2·0 ml. If several compounds were administered to the same animal they were, if possible, dissolved in the same solution, but where this was not possible the total volume injected did not exceed 2·0 ml. Control rats were injected with an equal volume of water. Rabbits were usually given 10 ml. (20 ml. for DL-serine). Pyruvic acid was neutralized with $NaHCO_3$ before dilution to the required volume.

Collection and deproteinization of tissues

Blood. This has been described previously (Simkin & White, 1957).

Other tissues. The method of Simkin & White (1957) was used when only glycine was determined, but this was modified, as suggested by Schurr, Thompson, Henderson & Elvehjem (1950), when filtrates were prepared for the estimation of serine by microbiological assay, in the following way. The frozen sample (3–4 g.) was heated with 10 ml. of water in a boiling-water bath for 3 min. The cooled sample was comminuted with a further 10 ml. of water and the requisite amount of tungstate mixture. Tungstic acid filtrates were stored under toluene in a refrigerator overnight and brought to pH 6·8–7·0 before assay.

Determination of serine

Serine was estimated by microbiological assay, with *Leuconostoc mesenteroides* P60 and a slight modification of the method of Henderson & Snell (1948). Sodium benzoate (final concn. 0·1 mM) did not interfere with the growth of the organism (cf. Nurmikko & Virtanen, 1951). Krueger (1950) used a similar method of assay and reported that β-hydroxybutyric and pyruvic acids had no effect upon the growth rate of this organism.

Plan of experiments

This was, in general, as described by Simkin & White (1957). The mean control value obtained from all experiments was used for rats. Microbiologically available serine has been termed 'free L-serine'.

RESULTS

The results obtained for the levels of glycine and serine in rat liver and blood and in rabbit blood are presented in Tables 1–3. Table 1 contains values obtained in rat liver after administration of glycine, Table 2 those after administration of serine and its precursors, and Table 3 those for levels of glycine and serine in rat and rabbit blood.

Administration of glycine

The intraperitoneal injection of glycine caused a rapid rise in the concentration of free glycine in rat liver (Table 1). The concentration of free L-serine in liver was similarly affected. The ratio of the concentration of serine to that of glycine remained approximately constant. The control value of this ratio was 0·45, and the mean value after administration of glycine was 0·46 (± S.D. 0·14, 8 values).

Table 3 shows the results of experiments on the effect of injection of glycine into the rat and rabbit. The blood glycine level increased about 12-fold at 0·5 hr. after injection. The concentration of free glycine in rat liver and in rat and rabbit blood increased also after simultaneous injection of equimolecular quantities of glycine and benzoate (Tables 2 and 3), but the increase was smaller than that observed when a similar dose of glycine was administered alone (Table 1). [A similar result was obtained by de Vries & Alexander (1948), who

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studied the free glycine concentration of human plasma.] Although the rise in glycine concentration observed was smaller than when glycine alone was administered, the concentration of free serine in rat liver still increased (Table 2).

Administration of serine and serine precursors

The concentration of free serine in the liver was raised to a considerable extent by administration of DL-serine, acetate, pyruvate and DL-β-hydroxybutyrate (Table 2). There was not a marked rise in the level of glycine. The ratios of the concentrations of serine to those of glycine were well above the control value (0·45) and ranged from a minimum of 0·75 after the administration of pyruvate to 1·40 after administration of DL-serine.

The concentration of serine, after administration of DL-serine + benzoate, was raised to approximately the same extent as in the absence of benzoate, and the administration of serine failed to prevent the decrease in glycine concentration normally found in the presence of benzoate (Simkin & White, 1957); the ratio of serine to glycine concentration was about 2·8.

After administration of glycine and benzoate the concentrations of both free L-serine and glycine in

Table 1. *Effect of intraperitoneal injection of glycine on the concentration of free glycine and serine in rat liver*

Each determination was carried out on the pooled livers from two rats. Mean control values for undosed animals were: free glycine, 268±42 S.D. (33 expts.); free (microbiologically available) L-serine, 120±9 S.D. (7 expts.). Column headings in hr. refer to time of death; doses were injected at 0 hr.

Expt.	Dose (μmoles/100 g. body wt.)	Free glycine (μmoles/100 g. of fresh tissue)					Free L-serine (μmoles/100 g. of fresh tissue)			
		0·5 hr.	1·5 hr.	2·0 hr.	3·0 hr.	5·0 hr.	0·5 hr.	1·5 hr.	3·0 hr.	5·0 hr.
A	360	980	—	530	—	—	—	—	—	—
B	190	655	—	355	—	—	—	—	—	—
C	380	835	535	—	525	420	—	—	—	—
D	460	1250	770	—	405	255	365	335	170	105
E	390	950	515	—	345	—	455	275	265	—
F	430	1160	—	—	—	—	410	—	—	—

Table 2. *Effect of intraperitoneal injection of serine and serine precursors on the concentration of free glycine and serine in rat liver*

Each determination was carried out on the pooled livers from two rats. Mean control values for undosed animals: free glycine, 268±42 S.D. (33 expts.); free (microbiologically available) L-serine, 120±9 (7 expts.). Column headings in hr. refer to time of death; doses were injected at 0 hr.

Expt.	Compound administered	Dose (μmoles/100 g. body wt.)		Free glycine (μmoles/100 g. of fresh tissue)					Free L-serine (μmoles/100 g. of fresh tissue)				
		Serine or precursor	Benzoate	0·25 hr.	0·5 hr.	1·5 hr.	3·0 hr.	5·0 hr.	0·25 hr.	0·5 hr.	1·5 hr.	3·0 hr.	5·0 hr.
G	DL-Serine	380	—	—	260	300*	—	—	—	—	—	—	—
H		410	—	300†	245‡	—	—	—	—	—	—	—	—
I		1000§	—	—	310	280‖	—	—	—	—	—	—	—
J		400	—	—	320	315	305	265	—	—	—	—	—
K		440	—	—	300	270	255	—	—	415	295	260	—
L	DL-Serine + benzoate	420	420	—	140	185	115	105	—	—	—	—	—
M		410	380	—	145	115	90	—	—	430	295	250	—
N	Acetate	400	—	180	—	—	—	—	195	—	—	—	—
O		1000	—	—	240	—	—	—	—	265	—	—	—
P	Pyruvate	400	—	215	—	—	—	—	165	—	—	—	—
Q		1000	—	—	210	—	—	—	—	190	—	—	—
R	DL-β-Hydroxybutyrate	400	—	260	—	—	—	—	230	—	—	—	—
S		1000	—	—	270	—	—	—	—	260	—	—	—
T	Glycine + benzoate	350	350	—	730	405	200	240	—	—	—	—	—
U		400	370	—	605	260	110	—	—	375	255	190	—
V	Benzoate	—	460	—	90	125	100	105	—	90	125	95	150
W		—	470	—	—	115¶	170	90	—	—	120¶	165	75

* 2 hr. † 0·35 hr. ‡ 0·75 hr.
§ Owing to the sparing solubility of DL-serine, 600 μmoles/100 g. of this dose were given by stomach tube.
‖ 0·9 hr. ¶ 1 hr.

Table 3. *Effect of intraperitoneal injection of serine, glycine and benzoate on the concentration of free glycine and serine in blood*

Each determination for rats was carried out on the pooled blood of two animals. Mean control values for undosed animals were: free glycine 20±4 S.D. (29 expts.); free (microbiologically available) L-serine 22±8 (4 expts.). For rabbits, blood samples were withdrawn at the stated times and the control value (0 hr.) for each animal is given. Column headings in hr. refer to time of death of rats, or time of taking sample from rabbits; doses were injected at 0 hr. Lettered experiments refer to rats and numbered experiments to rabbits.

Expt.	Compound administered	Dose (μmoles/100 g. body wt.) Glycine or serine	Benzoate	Free glycine (μmoles/100 g.) 0 hr.	0·25 hr.	0·5 hr.	1·5 hr.	3 hr.	5 hr.
C	Glycine	380	—	—	—	260	106	52	44
T	Glycine + benzoate	350	350	—	—	114	52	18	23
J	DL-Serine	400	—	—	—	19	18	17	17
L	DL-Serine + benzoate	420	420	—	—	10	12	9	11
1	Glycine	430	—	63	720	860	820	530	310
2	Glycine + benzoate	390	390	57	210	250	187	120	69
3	DL-Serine	300	—	107	165*	157	151	162	143
4	DL-Serine + benzoate	290	290	107	84*	74	47	27	16
				Free L-serine (μmoles/100 g.)					
V	Benzoate	—	460	—	—	20	16	17	16
W	Benzoate	—	470	—	—	10†	—	16	12
5	Benzoate	—	400	33	—	36‡	29	30	—

* 0·3 hr. † 1 hr. ‡ 0·75 hr.

the liver were increased initially above the control value in almost the same proportion, but subsequently the serine level decreased more slowly than the glycine level (Table 2). The ratio of serine to glycine concentration rose from 0·62 to 1·9. Administration of DL-serine did not result in any significant change in the concentration of free glycine in rat or rabbit blood (Table 3).

Administration of benzoate

The concentrations of free glycine and L-serine in rat liver after administration of benzoate are given in Table 2. There is no evidence for a large change in concentration of free serine, but since the variation (S.D. ± 32) appears to be greater than that in the controls (S.D. ± 9) no conclusion can be drawn as to the possibility of a small variation. The concentration of free glycine in the same experiments decreased, as is usual after benzoate administration (Simkin & White, 1957). The change in the concentration of free serine in the blood of a rabbit after the administration of benzoate was small (Table 3). Injection of serine with benzoate did not prevent the decrease in free glycine of the blood, which is characteristic of benzoate administration.

DISCUSSION

Formation of serine from glycine

The change in tissue levels of serine after administration of glycine show that there is a rapid net synthesis of serine from glycine, which indicates that the rate of formation of serine from glycine varies markedly with the tissue level of glycine. It had already been shown (e.g. by Arnstein & Neuberger, 1953) that glycine may provide both the hydroxymethyl group of serine and the remaining part, but investigations with labelled glycine did not exclude the possibility that the rate of the reaction was limited by a reaction step following zero-order kinetics. It is clear that this is not so, and that glycine from dietary sources can act as an effective precursor of serine.

This fact is of considerable interest in connexion with the glycogenic action of glycine. Although Hess (1950) found that glycogen was rapidly formed from administered DL-serine, there is a time lag of several hours before glycogen is formed from administered glycine (see Arnstein, 1954), and it has been suggested (Arnstein, 1954) that a possible explanation of this lag is the limited net rate of conversion of glycine into serine. The results obtained from the present investigation do not support this explanation.

Formation of glycine from serine

It is known that glycine is readily formed from serine, since administration of isotopically labelled serine results in the production of glycine with a specific activity as high as 63% of that of the administered serine (Elwyn & Sprinson, 1954). We have found, however, that the tissue levels of glycine after administration of serine showed little or no increase above normal values, which indicates that the rate of conversion of serine into glycine varies little with the tissue level of serine. An

increase in the tissue level of serine resulting from administration of nitrogen-free precursors also fails to change the tissue level of glycine, showing that the lack of effect of administered serine is not caused by permeability barriers. Moreover, when serine is formed from administered glycine, and the level of glycine is reduced by benzoate, the serine level decreases more slowly than that of glycine.

The present investigation shows that there is no evidence for an increase in the level of free glycine in liver after administration of serine or its precursors (see, however, Christensen, Streicher & Elbinger, 1948; Greenberg & Sassenrath, 1955). The mean of 19 values after administration of DL-serine, acetate, pyruvate or DL-β-hydroxybutyrate was 264 ± 37 S.D.; the mean of 33 control values was 268 ± 42. The fact that the value of the standard deviation of the mean free glycine level of liver after administration of serine or its precursors is very close to that of the control confirms that this calculation of the mean value of the determinations at different time intervals after dosage is permissible; calculation of such an average value would tend to obscure a temporary rise in the free glycine level of liver, but such a rise would lead to a large standard deviation. The mean of 12 values for free serine in liver was 270 ± 81 as compared with a control value of 120 ± 9 (7 expts.). The standard deviation of 81 corresponds to real differences in the effects of administering serine or its precursors, as well as to temporary changes in the level of free L-serine. The evidence shows that an increase in the tissue level of serine has much less effect on the conversion of serine into glycine than an increase in the tissue level of glycine has on the formation of serine. This may be expressed formally by saying that the kinetics followed by the serine $\rightarrow$ glycine reaction approach zero- rather than first-order. This is supported by the inability of DL-serine to restore the growth rate of glycine-depleted rats (White, 1941).

Availability of serine and glycine

The results of Arnstein & Neuberger (1953) show that the gross rate of formation of glycine from serine and other precursors is independent of the supply of glycine in the diet. As shown in the previous section, this rate is only slightly increased by the administration of serine. The present investigation shows that there is a net formation of serine from glycine after administration of glycine, which indicates that the gross rate of formation of serine increases with increasing glycine level.

These findings seem to suggest that in the absence of dietary sources of glycine the net production of glycine from serine will increase with the requirements of the organism for glycine. As the requirement for glycine increases, the tissue level of glycine will decrease. An extreme instance of this is the marked decrease in free glycine of the liver after administration of benzoate (Table 2). The rate at which glycine is converted into serine will therefore decrease, so that if the serine level stays constant the net production of glycine would be greater than before. The present results suggest, however, that the reduction in the glycine level after administration of benzoate does not affect the rate of formation of serine. It is known that the α-C atom of glycine is a source of the β-C atom of serine, presumably via formaldehyde or a derivative at the same level of oxidation (Elwyn & Sprinson, 1954). When there is a plentiful supply of glycine, up to 60% of the β-C atoms of serine is derived from glycine, but at normal glycine levels glycine acts as precursor of the β-C atom of serine to a negligible extent (Arnstein & Neuberger, 1953). Under these conditions, therefore, glycine is of little importance as a source of 'formaldehyde', and a lowering of the glycine level would not appreciably diminish the availability of 'formaldehyde'. The fact that serine levels are maintained even when the glycine level is depressed by benzoate suggests that the rate of formation of serine is limited by the concentration of 'formaldehyde' rather than of glycine.

SUMMARY

1. The influence of the administration of benzoate, glycine, DL-serine, acetate, pyruvate or DL-β-hydroxybutyrate on the concentrations of free glycine and free L-serine in the liver and blood of the rat and the blood of the rabbit has been investigated. Administration of glycine resulted in a rise in the concentration of both glycine and serine. Administration of serine or its non-nitrogenous precursors resulted in a rise in the concentration of serine but not of glycine. Administration of benzoate alone resulted in a fall in the concentration of glycine but not of serine.

2. The significance of the results is discussed with reference to the kinetics of the interconversion of glycine and serine.

We wish to thank Dr F. W. Norris for providing a culture of *Leuconostoc mesenteroides*. We are indebted to Dr W. V. Thorpe and Dr H. G. Bray for their interest and discussion of this work and to Mr P. B. Wood, Mrs B. G. Taylor and Miss D. A. Clarke for technical assistance.

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BBA 65312

TYROSINE-α-KETOGLUTARATE TRANSAMINASE
EFFECT OF THE ADMINISTRATION OF SODIUM BENZOATE AND RELATED COMPOUNDS ON THE HEPATIC ENZYME LEVEL

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(Received March 23rd, 1965)

SUMMARY

Intragastric administration of sodium benzoate caused increased levels of tyrosine-α-ketoglutarate transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) in the livers of intact and adrenalectomized rats. Maximal increase occurred 3 h after benzoate administration to adrenalectomized rats. The response increased as the benzoate dosage was varied from 5 to 20 mg per 150 g body weight, reaching a maximal level 4-fold greater than that of saline-injected controls. Higher dosage levels gave little if any further increase and in many cases resulted in toxic manifestations.

Thirty-one cyclic compounds were compared with benzoate as inducing agents. Hydrocortisone, hydrocortisone hemisuccinate and diethylstilbestrol disulfate were more effective than benzoate. Cyclohexanoate, *p*-aminobenzoate, and α-naphthoate were as effective. Benzene, phenol, α-naphthol, β-naphthoate, phenylacetate, α-naphthylacetate, nicotinate, α-picolinate, salicylate, *p*-nitrobenzoate, cyclohexane, pyridoxine, pyridoxoate, DL-phenyllactate, *o*-phthalate, terephthalate, *o*-diphenate, pyromellitate, isonicotinate, benzene sulfonate, pyridine 3-sulfonate, sulfanilate, anthranilate, *p*-hydroxybenzoate, and *o*-nitrobenzoate were either substantially less effective or were ineffective.

Strong inhibition of the increase by injected puromycin and actinomycin D, compounds which inhibit protein and RNA synthesis respectively, suggests that the benzoate-mediated effect occurred by a mechanism involving increases in protein and RNA synthesis. In this respect, the effect of benzoate resembles that of the glucocorticoids.

INTRODUCTION

The administration of glucocorticoids to intact or adrenalectomized rats causes within several hours an elevation of the activity of the hepatic tyrosine α-ketoglutarate transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5)[1]. Sub-

tial evidence supports the view that this increase reflects elevated levels of the enzyme in the liver[2]. Such an increase must arise from an increased rate of apoenzyme synthesis relative to its rate of destruction or to a decreased rate of destruction relative to its rate of synthesis. Since alterations in the rate of apoenzyme destruction following hydrocortisone administration could not be detected[3], the increased activity appears to be a result of an increased rate of synthesis. Consistent with this possibility, puromycin and ethionine, which are known to inhibit protein synthesis, strongly inhibit the induction[1-6]. Concomitant increases in the amount of RNA and of the rate of precursor incorporation into RNA[7,8], considered together with the inhibiting action of actinomycin D, an inhibitor of RNA synthesis, on the rate of induction[4,5], provide support for the view that the glucocorticoid effect may involve increased synthesis of some RNA species which is rate-limiting in enzyme synthesis.

Although a number of other compounds, apparently unrelated to the glucocorticoids, also caused increased activity of tyrosine transaminase when injected into intact rats[6,9], most of these were ineffective in adrenalectomized rats[6]. Of the various compounds tested in adrenalectomized rats, only pyridoxine[10], tryptophan[6], and several tryptophan derivatives[6] caused significant increases. Like the glucocorticoid-mediated inductions, these effects occurred within several hours after injection of the compounds and were inhibited by simultaneous injections of puromycin[6,10]. The action of pyridoxine was not inhibited by injected actinomycin, however, and in this respect was different from that of the glucocorticoids. This difference led to the view[10,11] that a mechanism different from that of the actinomycin-sensitive, "hormone-type" induction is involved. More specifically, it has been proposed[10,11] that pyridoxal phosphate, derived from injected pyridoxine, forms a complex with tyrosine α-transaminase and thereby increases its rate of synthesis, perhaps by "pulling" the reaction. A similar mechanism was suggested for the action of tryptophan and its derivatives[11]. The proposed mechanism, which was considered earlier for the induction of other enzymes[11,12], has been designated "substrate-type" induction[11].

The present report describes a study of the inducing action of sodium benzoate and some related compounds on the tyrosine-α-ketoglutarate transaminase of the livers of adrenalectomized rats. The possible relationship of these effects to those induced by other inducers* of the enzyme is discussed.

EXPERIMENTAL PROCEDURE

General procedure

Male albino rats, 6 weeks old and weighing initially 120–140 g, were obtained from the Holtzman Rat Company, Madison, Wisc. All animals received Rockland Rise Rat Food and water *ad libitum*. Bilateral adrenalectomy[13] under ether anesthesia was performed within one week of their arrival; 1% NaCl was substituted for the drinking water thereafter.

* The increased enzyme activity caused by non-steroid compounds has not been shown by rigorous criteria to be a result of increased apoenzyme concentrations. For convenience, however, we shall refer in this report to all administered compounds that cause increased activity as inducers, and to the increase as induction, unless kinetic factors other than apoenzyme concentration are known to be responsible for the increase.

After 7 days on 1% NaCl, randomly-picked animals were given water. This usually resulted in death within 2–3 days. Those rats surviving (about 10%) were found to have residual adrenal tissue. Visual examination revealed such adrenal "rests" in only 3–4% of the animals used in the present study; the data in these cases were discarded.

Seven or eight days after adrenalectomy, food was removed from the cages and, 18–19 h later, sodium benzoate or equivalent amounts of related compounds were administered by stomach tube. The rats were killed by decapitation, usually 3 h after injection, and the livers were homogenized in 4 times their weight of cold 0.14 M NaCl solution containing 0.02 M sodium phosphate (pH 7.6). The homogenate was centrifuged briefly at 30 000 × g at 5° and the supernatant fluid was then assayed immediately for tyrosine-α-ketoglutarate transaminase activity.

Assay procedure

The supernatant fluid was diluted 5-fold with cold distilled water and 0.30- and 0.70-ml aliquots of the diluted preparation were added to 1.10-ml portions of reaction mixture containing 22 μmoles α-ketoglutarate, 310 μmoles sodium phosphate buffer (pH 7.6), 14.5 μmoles disodium diethyldithiocarbamate, and 60 μg pyridoxal phosphate. After a 3-min preincubation at 37° with continuous shaking, the reaction was started by the addition of 24 μmoles of L-tyrosine dispersed in 2.4 ml of distilled water. At 0 and 10 min, the reaction was stopped by the addition of 0.40 ml of 100% (w/v) trichloroacetic acid. The deproteinized solutions were filtered and 2.00-ml aliquots of the filtrates were assayed for *p*-hydroxyphenylpyruvate by a modification[14] of the Briggs method. Absorbance at 660 mμ was compared with that of known amounts of *p*-hydroxyphenylpyruvate carried through the assay procedure. Results are expressed as the change of absorbance obtained with the 0.3-ml aliquot in 10 min. The absorbance values may be converted, to a close approximation, to μmoles of *p*-hydroxyphenylpyruvate formed per g of liver per 10 min by multiplying by 208.

Materials

Paromycin, pyridoxine hydrochloride, α-ketoglutarate, pyridoxal 5-phosphate, and L-tyrosine were purchased from Nutritional Biochemicals Corp.; actinomycin D was a gift from Merck, Sharp and Dohme. Benzoate and the related compounds were obtained from commercial sources. They were recrystallized as necessary, dissolved in physiological saline, and neutralized with NaOH solution to a final pH of 7.4. Unless stated otherwise, final concentrations of administered compounds were: paromycin, 20 mg/ml; actinomycin D, 1.0 and 0.1 mg/ml; sodium benzoate and related compounds, 0.175 M. All solutions were administered in volumes proportional to 1 ml of solution per 150 g body weight.

RESULTS

Effect of benzoate administration on liver tyrosine transaminase levels in intact and adrenalectomized rats

The effect, observed in intact rats, was a 2-fold elevation of the tyrosine transaminase activity 3 h after a 30 mg (per 150 g body weight) dose of sodium benzoate solution was administered by stomach tube. A 10 mg dose of hydrocortisone hemi-

TABLE I

INDUCTION OF HEPATIC TYROSINE TRANSAMINASE 5 h AFTER ADMINISTRATION OF SODIUM BENZOATE AND HYDROCORTISONE HEMISUCCINATE TO INTACT AND ADRENALECTOMIZED RATS

Physiological saline or sodium benzoate (50 mg per 150 g body weight) was injected at zero time by stomach tube; hydrocortisone hemisuccinate (10 mg per 150 g body weight) was injected at zero time intraperitoneally. Activity is expressed as the change in absorbance at 660 mμ occurring in 10 min. Data are given as the average ± standard deviation. To convert activity values to μmoles *p*-hydroxyphenylpuryvate formed, multiply by 208.

Administered	*Intact*		*Adrenalectomized*	
	Number of animals	*Activity*	*Number of animals*	*Activity*
Saline	4	0.183 ± 0.004	10	0.069 ± 0.008
Sodium benzoate	4	0.346 ± 0.029	10	0.167 ± 0.005
Hydrocortisone hemisuccinate	2	0.360 ± 0.002	3	0.508 ± 0.033

* Addition of 5 mg sodium benzoate or 7.5 mg sodium hippurate to the incubation media from similar saline controls did not significantly influence the enzyme activity. This suggests that the increased activity is not attributable to activation of the enzyme by benzoate or hippurate retained in the liver.

succinate intraperitoneally gave a similar 2-fold induction (Table I). Since this effect of benzoate could have been due to stimulation of adrenal secretion, the same experiment was carried out with adrenalectomized rats. Under these conditions, a 2.4-fold induction was observed with benzoate and a 7-fold induction with hydrocortisone hemisuccinate. The effect of adrenalectomy in increasing the response to corticosteroids has been reported previously[15].

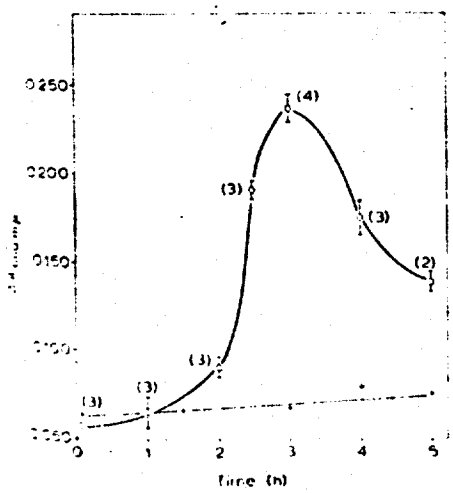

FIG. 1. The time course of the benzoate mediated induction of hepatic tyrosine transaminase. A single dose (50 mg per 150 g body wt.) was administered by stomach tube and the rats were killed after various time intervals as indicated. Numbers in parentheses indicate the number of rats used for each point. Open circles represent the average values for the induced series; the vertical lines show the standard deviations. Each cross represents a single control animal which received the same volume of saline but no benzoate.

Time-course and dose-response in adrenalectomized rats

The time-course of the induction by benzoate showed a maximal induction (4.2-fold) 3 h after benzoate administration (Fig. 1). After 5 h, the induction level had dropped to the 2-fold level observed earlier (Table I) for that time period.

Many of the rats given 50 mg of benzoate showed toxic effects, characterized by salivation, convulsions, and prostration. A dose-response curve (Fig. 2) was run in the hope of finding a benzoate level that would give substantial induction without apparent toxicity. Using the 3-h induction period, the response (level of induction) was found to be roughly proportional to dosage level in the range of 5–20 mg per 150 g body weight. The maximum induction level (4-fold) was obtained with 20-mg doses;

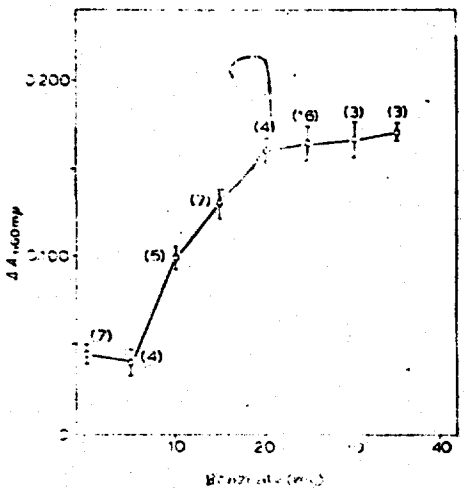

Fig. 2. Dose-response [illegible] the benzoate-mediated induction of hepatic tyrosine transaminase. The indicated doses (mg per 150 g body weight) were injected by stomach tube and the rats were sacrificed 3 h later. Numbers in parentheses indicate the number of animals used for each point. Each point represents the average of five values; vertical lines show the standard deviation.

large doses (25, 30 and 35 mg) gave approximately the same response, with prostration occurring at the 30- and 35-mg levels. In subsequent experiments, 25 mg per 150 g body weight was used unless otherwise indicated. Numerous experiments at these and lower levels have demonstrated that 3- to 4-fold inductions can be obtained consistently with benzoate and related compounds without visible effects on the appearance or behavior of the rats. Conversely, some substituted benzoates (anthranilic acid, for example) have caused prostration without causing significant induction in the time interval studied.

Comparison of the intragastric and intraperitoneal routes

Most of the studies of the induction of tyrosine transaminase by hydrocortisone and other compounds have utilized the intraperitoneal route of administration. Herman and coworkers[16,17] used the intragastric route to study several parameters of glucocorticoid action (including the induction of tyrosine transaminase) and found much weaker responses than those reported for equivalent intraperitoneal doses.

TABLE II

INDUCTION OF TYROSINE TRANSAMINASE IN ADRENALECTOMIZED RATS BY SODIUM BENZOATE ADMINISTERED INTRAPERITONEALLY AND INTRAGASTRICALLY

Activities are expressed as average absorbance values ± standard deviations. The numbers in parentheses indicate the number of animals used.

*Benzoate dosage** (mg per 150 g body wt.)	*Activity*		
	Saline	*Benzoate (i.g.)*	*Benzoate (i.p.)*
25	0.050 (i.g.) (1)	0.185 (1)	0.194 (1)
15	0.058 ± 0.003 (i.p.) (2)	0.137 ± 0.006 (3)	0.139 ± 0.004 (3)

* Physiological saline or sodium benzoate at the dosages indicated was given intraperitoneally (i.p.) or by stomach tube (i.g.) and the animals were killed 3 h later.

TABLE III

INHIBITION OF THE BENZOATE-MEDIATED INDUCTION OF HEPATIC TYROSINE TRANSAMINASE BY PUROMYCIN AND ACTINOMYCIN D

At zero time, all animals were injected by stomach tube with 1 ml of saline or 0.175 M sodium benzoate per 150 g body weight. In Expt. 1, this was followed in 15 and 90 min by intraperitoneal injection of 5 mg of puromycin in 0.25 ml of saline or of saline alone. In Expt. 2, 1 mg of actinomycin D in 1 ml of saline or saline alone was injected intraperitoneally at 15 min. Expt. 3 differs from 2 only in that 0.100 mg of actinomycin D, rather than 1 mg, was used. In each case, animals were killed 3 h after administration of benzoate or saline. Activity data are given as the average absorbance reading ± the standard deviation. The numbers in parentheses show the number of animals used. Fold induction shows the ratio of activities of treated animals to those of the appropriate saline-injected controls. The effect of inhibitors is represented as per cent inhibition of the increase in activity attributable to benzoate administration $\left(= 100\left(1 - \frac{B-1}{A-1}\right)\right)$.

Treatment	*Activity*	*Fold induction*	*% inhibition*
Experiment 1			
Saline	0.068 ± 0.007 (5)		
Saline + benzoate	0.286 ± 0.016 (3)	(A) 4.2	
Saline + puromycin	0.108 ± 0.010 (5)		
Saline + puromycin + benzoate	0.144 ± 0.003 (5)	(B) 1.3	90
Experiment 2			
Saline	0.047 ± 0.005 (4)		
Saline + benzoate	0.183 ± 0.030 (4)	(A) 3.9	
Saline + actinomycin	0.051 ± 0.006 (5)		
Saline + actinomycin + benzoate	0.090 ± 0.020 (5)	(B) 1.7	73
Experiment 3			
Saline	0.055 ± 0.005 (3)		
Saline + benzoate	0.200 ± 0.005 (4)	(A) 3.7	
Saline + actinomycin	0.043 ± 0.001 (3)		
Saline + actinomycin + benzoate	0.095 ± 0.003 (4)	(B) 2.2	56

This finding prompted us to compare the benzoate effects obtained by the two different injection routes. As shown in Table II, varying the route did not cause significantly different responses in 3 h with two different levels of benzoate. The intragastric route was used in most of the subsequent studies.

TABLE IV

RELATIVE ABILITIES OF BENZOATE AND RELATED COMPOUNDS TO INDUCE TYROSINE TRANSAMINASE IN THE LIVERS OF ADRENALECTOMIZED RATS

Experiments were run with sets of 4 animals, one injected with physiological saline, one with sodium benzoate (25 mg per 150 g body weight, unless noted otherwise), and two with the compound to be tested (an amount equimolar with benzoate, usually). The animals were sacrificed after 3 h and the enzyme levels determined. The results are expressed as the ratios of the averages of the activities in the induced and saline-injected animals. The relative effectiveness of each compound as compared to benzoate is indicated in column C and is calculated from data in columns A and B ($C = (B - 1)/(A - 1) \times 100$). Numbers in parentheses indicate number of animals used.

Compound	(A) Benzoate / Control	(B) Inducer / Control	(C) % Benzoate Induction
1 Benzene*,**	3.8 (2)	2.0 (4)	36
2 Phenol*	3.6 (2)	2.1 (5)	42
3 Benzoic acid	4.0 (6)	(4.0)	100
4 Cyclohexane	4.0 (2)	1.0 (4)	0
5 Cyclohexanoic acid	3.9 (2)	3.9 (4)	100
6 Pyridoxine	4.5 (2)	1.6 (6)	17
7 Pyridoxic acid*	3.2 (1)	1.5 (3)	23
8 α-Naphthoic acid	4.2 (2)	4.3 (4)	103
9 β-Naphthoic acid	4.3 (2)	2.5 (4)	46
10 Phenylacetic acid	3.8 (2)	2.7 (4)	61
11 α-Naphthalene acetic acid	3.9 (2)	2.8 (4)	62
12 DL-Phenyllactic acid	4.5 (2)	1.8 (3)	23
13 o-Phthalic acid	4.5 (2)	1.3 (4)	9
14 Terephthalic acid	3.9 (2)	1.3 (4)	10
15 o-Diphenic acid	4.2 (2)	1.3 (4)	10
16 Pyromellitic acid	3.6 (2)	1.2 (4)	8
17 Nicotinic acid	4.4 (2)	2.9 (4)	56
18 α-Picolinic acid	3.9 (2)	2.5 (5)	52
19 Isonicotinic acid	3.9 (2)	1.1 (4)	3
20 Benzene sulfonic acid	3.6 (2)	1.5 (3)	19
21 Pyridine-3-sulfonic acid	3.8 (2)	1.3 (3)	11
22 Sulfanilic acid	4.4 (1)	1.5 (4)	15
23 Tryptophan	3.8 (2)	2.3 (3)	48
24 p-Aminobenzoic acid	3.8 (2)	3.7 (4)	97
25 Anthranilic acid	3.9 (3)	1.5 (5)	17
26 p-Hydroxybenzoic acid	4.5 (2)	1.3 (4)	9
27 Salicylic acid	3.7 (4)	2.5 (5)	55
28 o-Nitrobenzoic acid	4.1 (2)	1.4 (4)	13
29 p-Nitrobenzoic acid*	3.6 (3)	2.4 (6)	54
30 Diethylstilbestrol disulfate***	3.6 (2)	1.6 (4)	23
31 Diethylstilbestrol disulfate***	3.8 (2)	3.7 (4)	97
32 Hydrocortisone***	3.8 (2)	5.3 (4)	155

* In autumn, a drop in the tolerance of the rats for sodium benzoate necessitated the use of 17-fold doses to avoid toxic symptoms.

** Benzene equimolar to 51 mg of sodium benzoate was used.

*** Doses of diethylstilbestrol disulfate and 5-mg doses of hydrocortisone (per 150-g body weight) were used. The greater induction with the estrogen sulfate (No. 31) was obtained in the intraperitoneal route of administration.

Inhibition of the benzoate-mediated induction of tyrosine transaminase by puromycin and actinomycin D

As mentioned earlier in this paper, two types of induction of tyrosine transaminase have been differentiated in adrenalectomized rats, a "hormone-type" that is inhibited by actinomycin D and a "substrate-type" that is not inhibited by actinomycin D. In an attempt to classify the benzoate effect, we administered puromycin and actinomycin D to rats receiving benzoate and studied the effect on the level of induction. The data (Table III) suggest that the response to benzoate resembles the hormonal response, since both puromycin and actinomycin D were strongly inhibitory. Experiments *in vitro* in which puromycin or actinomycin D were added directly to reaction mixtures showed no inhibition of the enzyme. Similar levels of actinomycin D did not cause inhibition of the inducing action *in vivo* of pyridoxine on tyrosine transaminase in the studies of GREENGARD AND GORDON[10].

Induction of tyrosine transaminase by compounds structurally related to benzoate

The 4-fold induction of tyrosine transaminase by benzoate is weaker than that reported for much smaller intraperitoneal doses of hydrocortisone. It seemed possible, therefore, that examination of compounds structurally related to benzoate might yield more potent inducers and perhaps provide information concerning the structural characteristics common to the various inducers. Accordingly, a variety of such compounds were tested and compared with benzoate (Table IV). Several (cyclohexanoate, α-naphthoate, and *p*-aminobenzoate) gave inductions approximately equal to that of benzoate, others (phenylacetate, α-naphthaleneacetate, *p*-nitrobenzoate, L-tryptophan, nicotinate, α-picolinate, salicylate, and β-naphthoate) gave approx. 2.5-fold inductions, while most had little or no effect. Diethylstilbestrol disulfate was an effective inducer when given by the intraperitoneal route but not when given by stomach tube.

DISCUSSION

KNOX[11] has suggested that the "substrate-type" induction of liver tyrosine transaminase in adrenalectomized rats by pyridoxine, tyrosine, tryptophan, serotonin, and 5-hydroxytryptophan may be dependent upon specific complex formation between inducers and enzyme. According to this concept, induction would occur as a result of the binding of the enzyme by its substrates (tyrosine and tryptophan), coenzyme (pyridoxal phosphate) or inhibitors (serotonin and 5-hydroxytryptophan). Benzoate and related compounds, which have some structural similarities to the aromatic substrates, might also be expected to bind tyrosine transaminase. Some have indeed found to be inhibitors of partially purified tyrosine transaminase from mouse[11] and rat[19] liver.

Of the various compounds tested, no clear-cut relationship between structural characteristics or potency as inhibitors and ability to induce was observed. Differences in the levels of induction may reflect differences in the levels of the different compounds in the liver rather than intrinsic differences in ability to interact with the cellular constituents. Since information concerning the intracellular concentration of each compound is lacking, precise structure function relationships cannot be obtained. It may be of interest, however, that all compounds found in the present study to

induce tyrosine transaminase possess as a common structural feature a fairly large planar hydrocarbon moiety. This characteristic is also common to those compounds (glucocorticoids, pyridoxine, tryptophan, and tryptophan derivatives) that were found to induce the enzyme in previous studies with adrenalectomized rats, whereas it is lacking in various compounds (amino acids, for example) found to be ineffective.

In addition to their possession of sizable hydrocarbon moieties, most of the inducers tested in this study also contained carboxyl groups. Some induction was also observed with benzene sulfonate, benzene, phenol, and diethylstilbestrol disulfate, however, so carboxyl groups are not essential for the effect. On the other hand, the present data do not eliminate the possibility that anionic groups of one type or another, either present in the administered compound or formed from it metabolically, may be important in causing inductions. A role for a metabolically-formed ester phosphate group (in pyridoxal phosphate) has in fact been assumed in rationalizing the inducing action of injected pyridoxine[10]. In view of the present results, which show induction by pyridoxic acid and various pyridine carboxylic acids, the possibility should be considered that other metabolic derivatives of pyridoxine (pyridoxic acid, for example) might mediate its effect.

A role for anionic groups in causing the inductions may also be inferred from the greater potency of benzoate and cyclohexanoate as inducers as compared with that of benzene and cyclohexane. The moderate inducing action of relatively large doses of benzene could possibly arise from its metabolic conversion to phenol which could, in turn, become anionic by conjugation to form the ester sulfate[19]. Consistent with this possibility, phenol at a much lower dose level was just as effective as benzene, whereas cyclohexane, which did not yield significant amounts of sulfate conjugates *in vivo*[19], was inactive.

The relatively potent effect of diethylstilbestrol disulfate deserves special comment. Its action was much greater when administered by the intraperitoneal route, in contrast to the approximately equal effects of benzoate given by the intragastric and intraperitoneal routes. These differences illustrate the importance of anatomic factors in influencing the processes under investigation. The potency of the estrogen sulfate is not necessarily attributable to the presence of the ester sulfate groups since these may perhaps be lost by hydrolysis prior to the penetration of the estrogen into the liver. On the other hand, even if hydrolysis did occur before penetration, sulfate esters might again be formed by sulfurylating enzymes known to exist in the liver[20]. Glucocorticoids, incidentally, are also converted in part into ester sulfates in rats[21] presumably to a large degree by the liver.

Although the induction of tyrosine transaminase by benzoate fits the concept of "substrate-type" induction in some respects, it is similar to the "hormone-type" in being inhibited by actinomycin D. Further studies may reveal the degree to which benzoate and related compounds resemble the glucocorticoids in their other actions. It is well-known that salicylate and various substituted benzoates and naphthoates mimic some of the actions of the glucocorticoids, notably their anti-inflammatory effects and certain metabolic effects believed to be associated with the anti-inflammatory action[21,22]. Anthranilic acid was reported to prolong the lives of adrenalectomized rats[23]. Small doses of 3-hydroxyanthranilic acid were found to stimulate the incorporation of labeled amino acids into liver protein 4 h after injection into fasted, intact rats[24], an effect similar to that reported for injected glucocorticoids[25]. In addition,

experiments, now in progress, further information will be sought concerning relationships between the actions of benzoate and related compounds and those of the glucocorticoids.

ACKNOWLEDGEMENT

This investigation was supported in part by a grant (AM-02294-07) from the U.S. Public Health Service.

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J. Ind. Hyg. Toxicol. 30(1):63-68, 1948.

FURTHER EXPERIENCE WITH THE RANGE FINDING TEST IN THE INDUSTRIAL TOXICOLOGY LABORATORY*

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THREE years ago we presented a paper (1) describing the Range Finding Test, which quickly and economically estimates the magnitude of some of the important health hazards of new chemicals. So far as we are aware and much to our surprise, there has been an absence of criticisms. We must confess we had temerity in setting forth such a simplified procedure in an age of growing refinements in techniques. A philosophy similar to ours has been admitted by Bratton (2), who describes a seven-day feeding test with mice which he employed with satisfaction for the preliminary examination of antimalarial agents. Recently one of us has had occasion to explain in some detail a program for the study of new chemicals, and to show the relation of the range finding test to this activity (3).

Far from weakening our faith, the passage of time has increased the value which we attach to this collection of abbreviated procedures. It is only by the use of short-cuts that our group has been able to accommodate in its schedule a reasonable amount of work which we consider advanced, to distinguish it from investigations of the caliber of the range finding test. The latter fills in our repertory a very real need for predicting the order of magnitude of the toxic hazards to be encountered in the production and utilization of a new chemical. The predictions may be sufficiently clear and favorable so that the compound is obviously no more hazardous to produce than a very familiar product; or so unfavorable that it is not manufactured until specially designed equipment can be obtained for synthesizing it safely; or the manufacture may be postponed because the applications which are foreseen for the product would be too hazardous in view of its physiological properties. But always it must be borne in mind that Range Finding data are in no sense final or complete, they must be supplemented by advanced studies before wide application of a chemical can be known to be safe. Our series of papers on the polyethylene glycols (4), not yet finished, represents rather concretely the concept of the many-sided advanced study.

Our earlier paper (1) stressed the speed with which sound approximations can be obtained by the range finding test. A period of three weeks was named as the time required for completion of the work. This estimate implied the existence of a staff ready to function upon receipt of a sample, and supplied at all times with suitable animals. In special situations the period elapsing before useful information is available can be much reduced. Within the past few years reassuring or cautionary statements have been issued as promptly as twenty-four hours after a sample was received, later to be amplified at greater leisure. Experience has now shown that such celerity is seldom required, and that it is uneconomical, if not actually impossible, to maintain a laboratory prepared to commence all the procedures of the range finding test the moment an express shipment is received.

Experience has further revealed that in the majority of cases some of the steps of the test may be omitted without prejudicing the applicability of the results to the immediate need. More and more as time passes, the steps performed are tailored to fit the specific need. A chemical may have such a low vapor pressure that inhalation is obviously of no concern to the industrial hygienist, a sample may be so scant that only those tests requiring minimum volumes can be performed, or the question accompanying the sample may be so specific that only one operation is required to obtain an answer. Happily this latter situation arises more often each year as those who originate requests grow more familiar with the nature of toxicological data and their own needs for information. The all inclusive question, "What is the toxicity of such and such a compound?", which would require a life time to answer, has almost vanished from our correspondence.

There are few standardized procedures which remain unchanged over several years and the details of the range finding test have never been regarded as more than tentative. It is not sur-

*Received for publication June 28, 1947. A paper presented at the 8th annual meeting of the American Industrial Hygiene Association, Buffalo, New York, on April 29, 1947.

prising therefore, that if the test were to be reduced to formal specifications today, it would be found to differ in several perhaps important respects from the description given in 1941. The differences will be discussed under the head of each separate step in the collection.

Single Dose Oral Toxicity

During the war our supply of Wistar strain rats was eliminated by the drafting of a commercial breeder. Comparison of several available strains allowed us to select the Sherman as an approximation to the Wistar in resistance to the acute toxicity of some of the more important chemicals which concerned us. Accordingly, the loss of the Wistar strain did not vitiate our files of acute toxicity data.

The LD_{50} is still bracketed by the use of groups of 6 rats given dosages differing in a ratio of 10. But now the approximation is often made closer by interpolating two groups of 10 rats, each of which suffers fractional mortality. Our standard dosage series, when expressed in gm./kg., constitutes the antilogarithms of 1.0, 1.1, 1.2, etc., and for range finding tests alternate dosages in the series are employed; the larger therefore exceeds the smaller by 58 per cent. These additional animals do not materially increase the time required for the test.

It is recognized that dependence upon one species is hazardous, accordingly for advanced studies other species are added.

Sub-acute Oral Toxicity

One of the serious deficiencies of the earlier description was the absence of any criterion of chronic toxicity. This defect has been remedied by administering doses to groups of 10 rats each, 5 of each sex in one cage, for thirty days. The sample is incorporated in a diet of 1000 gms. ground whole wheat, 500 gms. dried whole milk, and 20 grams of iodized salt, freely available to the animals in modified McCollum feeding cups from which very little is spilled. Water is provided in siphon drinking bottles and rarely it, rather than the diet, is the carrier of the chemical. The diet for each cage is kept in a separate jar which is weighed before and together with uneaten food in the cups after the thirty-day period. Three or four dosage levels differing by a ratio of 2 or 4 are administered, together with a control diet.

The criteria of injury in this step are effects on the increase in body weight from the approximate 120-gram starting weight, reduction in appetite, death, and micropathology of adrenal, upper intestine, kidney, liver and spleen. Table 1 sets forth typical results from such a test. It is apparent from the data that dosages were selected with the aim of allowing at least one group of rats to survive without injury as judged by the limited but sensitive criteria, rather than causing many fatalities. If the latter goal is pursued many rats will voluntarily starve themselves owing to the objectionable taste of diets containing high levels of chemical, and one will be uncertain of the cause of death. Daily forced feeding would avoid this doubt but would entail too much labor to be justified in a range finding test. Usually pathological study adds little quantitative information to the other observations, and it is doubtful if the operation is worthwhile unless the thirty-day feeding is regarded as a preliminary step to a two-year study where advanced work on a chemical justified the expense.

Planning of dosage levels is simplified by the observation that, under the conditions specified, a level of 1 per cent in the diet results in an average dosage of close to 0.6 gm./kg./day and 1 per cent in the water produces 0.8 gm./kg./day.

Skin Absorption

The factor of absorption through the intact skin grows more important each year. The previous communication (1) noted a shift from the four-day poultice upon guinea pigs to the one-day rubber cuff application upon rabbits of the Food and Drug Administration (5). The change is now complete and poultice data are regarded as obsolete because the saturated pad prevents complete contact of the dose with the skin. Dosages as great as 20 ml./kg. can be retained by the cuff, or dam, as it is known to us. The numbers of animals used are similar to those given oral dosages.

Vapor Exposure

The estimation of hazard by inhalation of saturated vapors has been amplified by adding the inhalation of vapors of known concentration, aiming at a concentration which produces fractional mortality as a result of a four-hour exposure. Concentrations used fall in a series differing by a ratio of 2, and they are approximations because they are estimated from the settings of a proportioning pump and flowmeter rather than being determined analytically. The latter procedure is

TABLE 1

Typical Results with Range Finding Test for Sub-Acute Oral Toxicity

Materials Administered in the Food to Groups of 10 Rats for 30 Days on each Dosage Level Unless Noted Otherwise

Material Studied	Range of Dosage Levels Fed		Least Daily Dosage Causing Symptom				Maximum Dosage Having No Effect	Single Dose Oral LD_{50} (2)
	Maximum	Minimum	Reduced growth	Reduced appetite	Micropathology (1)	Death		
	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.	gm./kg.
Acetophenone	0.102	0.001	—	—	—	—	>0.102	0.90 (0.81 to 1.00)
Acetylethanolamine	1.93	0.30	—	—	—	—	>1.93	27.66 (25.24 to 30.31)
Antimony trioxide	1.07	0.06	1.07	1.07	1.07	—	0.27	>20
Butyl "Carbitol" (3, 4)	1.83	0.051	—	0.094	0.65	—	0.051	6.56 (5.47 to 7.86)
Butyl phthalyl butyl glycolate	1.76	0.10	1.56	—	1.56	—	0.45	14.64 (11.73 to 18.28)
Calcium ethyl acetoacetate	0.34	0.014		—	0.060	—	0.014	9.93 (9.22 to 10.70)
"Carbitol" solvent (3, 4)	3.20	0.41	1.83	3.20	0.41	—	<0.41	9.05 (8.43 to 9.72)
"Carbitol" acetate (3, 4)	3.20	0.23	3.20	1.80	0.23	3.20	<0.23	11.00 (10.40 to 11.59)
Diacetone alcohol (3)	0.13	0.01	—	—	0.04	—	0.01	4.0 RF
Dibutyl "Cellosolve" phthalate	1.74	0.11	1.74	1.74	1.74	—	0.50	8.38 (6.86 to 10.23)
Diethylbutyl "Cellosolve" succinate	1.44	0.17	1.44	1.44	0.44	0.17	<0.17	3.3 RF
Diethylene glycol ethyl ether (pure) (3, 4)	3.88	0.21	1.77	0.87	1.77	—	0.49	8.69 (7.25 to 10.41)
Ethoxy triethylene glycol (3)	3.30	0.18	3.30	3.30	3.30	3.30	0.75	10.61 (9.98 to 11.28)
Ethylhexanimido diethyl (diethyl hexanoate)	1.53	0.10	—	—		—	>1.53	>39.8
Glycidyl oleate	0.35	0.014	—	—	—	—	>0.35	3.52 (3.35 to 3.69)
Heptadecyl glyoxalidine hydrochloride (3)	0.26	0.06	0.16	—	0.26	0.26	0.06	1.97 (1.70 to 2.32)
Methyl Acetyl Ricinoleate	1.82	0.12	—	—		—	>1.82	>50
Methyl "Carbitol" (3, 4)	1.83	0.19	1.44	0.79	0.19	—	<0.19	9.21 (7.90 to 10.71)
Methyl "Cellosolve" acetyl Ricinoleate	1.50	0.11	1.50	1.50		—	0.45	20.27 (15.13 to 27.15)
Methyl phthalyl ethyl glycolate	1.90	0.11	—	—	1.90	—	0.48	9.06 (8.11 to 10.13)
Propylene glycol ethyl ether (beta isomer) (3)	2.14	0.16	2.14	2.14			0.68	8.93 (7.89 to 10.09)
Sodium benzoate	1.09	0.016	—	—			>1.09	4.07 (3.72 to 4.44)
Sodium sorbate	1.08	0.016	—				>1.08	7.16 (6.82 to 7.52)
Sorbic acid	1.27	0.011	0.048		0.048	—	0.011	7.36 (6.69 to 8.90)
"Tergitol" penetrant 08 (3, 4)	1.51	0.23	1.24	1.24	0.80		0.44	10.32 (9.05 to 11.45)
"Tergitol" penetrant 4 (3, 4)	0.73	0.22	0.35	0.35	0.22		<0.22	4.95 (4.42 to 5.53)
"Tergitol" penetrant 7 (3, 4)	0.74	0.25	0.55	0.36	0.41		0.25	5.66 (4.85 to 6.60)
Triisopropanolamine (3, 4)	1.35	0.14	1.35	1.35	0.26		0.14	6.50 (5.62 to 7.54)
Triethylhexyl phosphate	1.55	0.11	1.55				0.43	37.08 (33.70 to 40.79)

(1) Dosage level producing micropathology in liver, kidney, spleen or testis of any rat.

(2) When LD_{50} was derived from an advanced test the range of 1.96 times the standard deviation is shown in parentheses. The letters "RF" refer to data derived from a range finding test. The symbol ">" indicates that the dosage shown is the maximum which could be administered and it killed none of 6 rats.

(3) Dose administered in the drinking water. In this case appetite is judged on the basis of ml. of water consumed.

(4) Only 5 rats to a dosage level.

TABLE 2

Typical Results with Range Finding Test

Capital Letters Designate Chemicals Having the Respective Hazard in the Same Order of Magnitude as the Material Studied, and Refer to the List Following the Table

Material Studied	Single Dose Oral Toxicity to Rats		Penetration of Rabbit Skin*		Saturated Vapor Inhalation by Rats		Inhalation of Known Vapor Concentration by Rats				Primary Skin Irritation	Eye Injury from Fluid	
	R.F. LD_{50}		R.F. LD_{50}		Maximum for no death		Concentration	Time	Mortality in 14 days			Grade	
	gm./kg.		*ml./kg.*				*PPM.*	*hrs.*					
Acetol	2.2	A	>16.0	U	—	—	2000	8	0/6	O	V	5	V
Acetonitrile	3.8	E	5.0	O	—	—	8000	4	1/6	Q	V	5	V
Acrylonitrile	0.093	N	(0.25)	N	1 min.	A′	500 1000	4 4	0/6 6/6	L	V	4	U
Allyl alcohol	0.064	N	0.053	A′	—	—	1000	1	4/6	A′	V	5	V
Allyl chloride	0.7	B	2.2	L	—	—	2000	4	1/6	A	V	2	Z
Allylidene diacetate	0.25	A′	0.32	N	—	—	8	4	4/6	T	C′	10	W
Chloropropane diol—1,3	0.11	N	0.45	B	—	—	1000	1	5/6	N	V	4	U
Chlorostyrene	5.2	O	20.0	Z	8 hr.	Z	—	—	—	—	U	2	Z
Cyanomethyl acetate	0.032	>N	0.043	A′	—	—	16 32	4 2	0/6 6/6	T	V	5	V
Diacetone alcohol	4.0	O	14.5	Z	8 hr.	Z	—	—	—	—	V	5	V
Dichloroethyl ether	0.075	N	(0.3)	N	—	—	1000	¾	3/6	A′	U	4	U
Dichloroethyl formal	0.065	N	(0.17)	N	—	—	60 120	4 4	0/6 6/6	N	V	1	Z
2,3-Dichloropropanol	0.09	N	0.2	N	—	—	500	4	2/6	L	U	5	V
Dichlorostyrene	4.0	O	9.0	I	8 hr.	Z	—	—	—	—	T	5	V
Dimethyl dioxane	3.0	O	>10.0	Z	—	—	8000	4	2/6	Q	B′	5	V
Dimethyl formamide	7.0	J	5.0	O	4 hr.	I	—	—	—	—	B′	4	U
Epichlorohydrin	0.09	N	1.3	A	—	—	250	8	4/6	A′	U	4	U
Ethoxy propionaldehyde	0.9	B	1.0	A	—	—	500	4	6/6	A′	V	7	N
Ethoxy propionic acid	4.8	E	0.75	A	—	—	2000	4	0/6	A	C′	9	W
Ethyl chlorobenzene	5.0	O	17.5	Z	8 hr.	Z	—	—	—	—	B′	2	Z
Ethyl dichlorobenzene	5.5	O	14.2	Z	8 hr.	Z	—	—	—	—	C′	1	Z
Ethylene imine	0.015	>N	(0.014)	A′	—	—	50 250	8 2	5/6 3/6	T	T	7	N
Ethylidene acetone	0.32	A′	0.5	B	—	—	250	4	3/6	A′	U	8	B
Hexane diol—2,5	5.0	O	16.3	Z	8 hr.	Z	—	—	—	—	V	3	S
Isopropanol	5.84	V	16.4	Z	—	—	16000	8	4/6	Q	—	4	U
Methyl acetoacetate	3.0	O	(>10)	Z	8 hr.	Z	—	—	—	—	U	7	N
Methyl acrylate	0.3	A′	1.3	A	—	—	1000	4	3/6	L	U	4	U
Methylbenzyl "cellosolve"	2.29	A	5.0	O	4 hr.	I	—	—	—	—	—	3	S
Methyl "cellosolve" acetate	3.39	O	5.25	O	—	—	7000	4	2/6	Q	—	2	Z
Methyl diacetoacetate	1.7	A	(>10)	Z	8 hr.	Z	—	—	—	—	C′	5	V
2-Methyl pentane-2,4-diol	4.70	O	13.30	Z	8 hr.	Z	—	—	—	—	U	4	U
Morpholine ethanol	12.06	S	(2.5)	L	8 hr.	Z	—	—	—	—	V	5	V
Propylene imine	0.019	>N	(0.043)	A′	—	—	500	4	5/6	L	—	9	W
Propylene oxide	1.14	B	(8.64)	I	—	—	4000	4	6/6	A	V	5	V
Tetramethyl ethylene diamine	1.58	A	7.0	I	10 min.	L	—	—	—	—	B′	8	B
Vinyl acetate	2.92	O	2.5	L	—	—	4000	4	3/6	O	V	2	Z

* Figures in parentheses were determined by poultices on guinea pigs.

COMPARATIVE MATERIALS REFERRED TO BY LETTER CODE IN TABLE 2

The words "Cellosolve" and "Carbitol" are trade marks of Carbide and Carbon Chemicals Corporation for the solvents that are ethers of ethylene and diethylene glycol respectively. These letter codes have the same significance as in our 1944 article (1). The symbol > before a letter in the table means significantly more toxic than

A	Methyl "Cellosolve" solvent	S	Ethanol
B	Formaldehyde	T	Dimethyl sulfate
E	Lactic acid	U	Ethyl acetoacetate
I	"Cellosolve" acetate solvent	V	Acetone
J	Triisopropanolamine	W	Ammonium hydroxide (28% NH_3)
L	Mesityl oxide	Z	"Carbitol" solvent
N	Ethylene chlorhydrin	A′	Crotonaldehyde
O	"Cellosolve" solvent	B′	Benzene
Q	Cyclohexanone	C′	Diethanolamine

impractical if a new vapor is to be handled every day.

At times there is appraised the hazard of certain operations which result in the creation of a mist by bubbling air through the sample held at the arbitrary temperature of 170° C., cooling the air, and furnishing it to animals in a small chamber. Limited data obtained in this way have been published (6). An attempt is being made to standardize the creation of a fog by the use of a nebulizer, but uniform conditions have not yet been attained. This exposure would reduce the oxidation of samples, which may be important in the 170° mist test.

SKIN IRRITATION

Tests of skin irritation are very difficult to standardize and quantitate, and no further progress in this direction has been achieved.

EYE INJURY

A full description of the eye injury test has recently appeared (7) together with a large amount of data, and no time will be spent upon the procedure here.

SKIN SENSITIZATION

A second deficiency recognized in the range finding test was the absence of any test for sensitization. During the past two years the Landsteiner guinea pig skin sensitization procedure has been studied. After standardization of an intradermal technique which appeared to yield reliable results, the test was applied to some 40 chemicals of widely different compositions. For at least half of these chemicals there were available for comparison the results of human patch tests, human experience, or experience with closely similar molecules. The results were disappointing. It was found that the guinea pig test did not miss chemicals which were active human sensitizers but that it did condemn others which have been used for years upon human skins without objectionable reactions. It was found to cost as much to perform as a human patch test series done by a consulting dermatologist and to require as much time. The guinea pig test therefore offers no advantages over human application and is no longer regarded as a part of the range finding test.

Materials for which preliminary skin sensitization data are desired are assembled in groups of 8 and referred to a consulting dermatologist who applies them to 30 human subjects. The schedule is one week of contact, about two weeks for development of sensitivity, and two to three days second contact to detect sensitization.

DISCUSSION

Table 2 presents examples of the data obtained with the range finding test. Skin sensitization has not been included and sub-acute oral data are in Table 1. It will be seen that we still lean heavily upon comparisons with more familiar molecules. This part of the test is perhaps the most indefensible because of pharmacological differences between even close analogs. But it is so valuable in allowing industrial technologists to visualize the meaning of toxicological information in terms of chemicals they handle every day, that the comparison is indispensable.

It must be emphasized that the range finding test is not believed to yield final or complete toxicological data. Its results are sound indications, but they remain tentative until confirmed and extended. The test can condemn a particular

application of a chemical but it cannot establish its safety. It must also be emphasized that the components are separable, and that rarely are all the steps performed upon a single material. The parts selected depend upon the information desired.

Summary

There are presented elaborations of the Range Finding Toxicity Test in respect to sub-acute toxicity, vapor inhalation, and skin sensitization. Further examples of the use of the test are given.

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The Effect of Combining Sodium Benzoate With Oral Penicillins[1]

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Diodrast (*8*), para-aminohippuric acid (*1*, *6*), benzoic acid (*3*), and sodium benzoate (*2*) have been reported as effective in elevating and prolonging penicillin serum levels, possibly by competition for renal tubular excretion. Bronfenbrenner and Favour (*3*) obtained at least a 2-fold increase by combining benzoic acid orally with intramuscular injections of sodium penicillin. Bohls and co-workers (*2*), using a total dose of 8.4 grams of sodium benzoate orally and a mixture of aluminum potassium sulfate and penicillin intramuscularly, reported assayable serum levels 28 hours following a single 50,000-unit injection. These same authors made determinations on an oral tablet containing alum-precipitated penicillin and sodium benzoate. The published data show assayable levels in 6 out of 10 individuals 24 hours after a single 100,000-unit dose. Both groups of investigators used infected individuals as subjects.

During the course of studies on oral penicillin the authors examined several preparations in combination with sodium benzoate. The present communication deals primarily with the effects observed. The subjects were 10 healthy adults who, with one exception to be mentioned later, participated in the entire study. Neither the diet nor the fluid intake was restricted; but food and fluids were avoided for a minimum of 1½ hours before administration of penicillin. The compounds were tested at one-week intervals. In all instances the test dose was 100,000 units of penicillin either alone or together with 1.2 gram of sodium benzoate. Blood was collected ½, 1, 1½, 3, and 6 hours later for serum level determinations, which were carried out by the method of Randall, *et al.* (*9*) and controlled for antisubtilis factor as described by Chandler and co-workers (*4*).

[1] These studies were made possible by financial aid from Hynson, Wescott and Dunning, Inc., and Commercial Solvents Corporation.

The authors gratefully acknowledge the technical assistance of Catherine C. Dietz and Cecelia Chemerda.

The crystalline sodium salt[2] used in these studies was reconstituted in water from the dried state. The aluminum salts[3] were received as unbuffered tablets with and without sodium benzoate. The alum-precipitated, penicillin-sodium benzoate tablet is identical with that reported on by Bohls and co-workers (*2*). The crystalline potassium penicillin was contained in gelatin capsules[3] and, when indicated, was taken at the same time as, but separately from, the sodium benzoate tablets.

TABLE 1

AVERAGE SERUM CONCENTRATION IN UNITS/ML.

Penicillin salt	Hours following administration (100,000 units)					
	½	1	1½	3	6	Average
Sodium	0.038	0.018	0.012	0.003	*	0.014
Aluminum	.009	.015	.015	.006	*	.009
Aluminum + sodium benzoate	.078	.101	.090	.027	.003	.060
Alum-ppt. + sodium benzoate	.054	.094	.094	.027	.003	.054
Potassium	.050	.040	.021	.009	*	.024
Potassium + sodium benzoate	.100	.136	.062	.024	.012	.067

* No assayable level with any of the 10 subjects.

The average serum concentrations for the 10 subjects appear in Table 1. To facilitate comparisons, the over-all averages for each preparation are also presented. In the absence of sodium benzoate none of the penicillin salts produced an average level above 0.05 unit/ml. or an assayable level at 6 hours. In contrast, the simultaneous administration of sodium benzoate resulted in maximum averages approximating or exceeding 0.1 unit/ml. and, in some instances, assayable levels at 6 hours.

The striking variation in individual response deserves emphasis. When averages were computed from the levels produced by each subject for all six preparations, it was found that the figures ranged from .009 to .073, an 8-fold difference. One subject did not show a single assayable level when the benzoate salt was omitted. In contrast, another subject accounted for all of the 6-hour levels recorded in Table 1. In passing, it should be mentioned that a subject who produced relatively high levels with one preparation also did so with the remaining preparations. Similarly, the "poor absorbers" remained relatively low, regardless of the preparation being employed. Because individuals vary markedly in their ability to absorb orally administered penicillin, the indiscriminate use of any one such preparation is not justified until it can be demonstrated that none of a large number of test subjects fails to develop therapeutically effective serum levels.

The inaccuracy of serum penicillin determinations has been repeatedly emphasized by the authors cited earlier. Although the alum-precipitated, penicillin-benzoate tablet included in the present study is apparently identical with that employed by Bohls and co-workers (*2*), the high and prolonged levels reported by those authors were not seen. As an explanation for the discrepancy it should be pointed out that their subjects were infected persons who might be expected to show higher levels than normal individuals and who are reputed to produce larger amounts of antisubtilis factor (*5*). Certain of the prolonged levels reported by Bohls could have been due to antisubtilis factor which was apparently not taken into consideration. With respect to the present study, however, no instance of complete inhibition was observed in the clarase controls.

The present authors were impressed by the need of close inspection of the tests for evidence of growth. The test organism (*Bacillus subtilis*, N.R.R.L. #558) usually developed in broth (2 per cent tryptose extract) as a sediment with some diffuse turbidity rather than as a pellicle. Careful observation often revealed a small amount of sedimented growth or a faint turbidity. Final readings were made after 21–24 hours incubation, and the levels were frequently lower than those recorded 5 hours earlier. Therefore, a standardized incubation time is desirable.

The ability of sodium benzoate to increase and prolong serum levels appears to be established. The choice of a dose of 1.2 gram/100,000 units, as employed in the present study, was purely arbitrary. In view of the relatively innocuous nature of this compound (*7*), the value of using larger amounts in combination with oral penicillin should be determined.

One subject in the present study developed a reaction which was attributed to penicillin sensitization. Fifteen minutes after the administration of the third compound urticaria appeared, followed later by edema. No other untoward effects were noted.

[2] Kindly supplied by Dr. L. W. Smith, of Commercial Solvents Corporation.

[3] Kindly supplied by Dr. Roger Reid, of Hynson, Wescott and Dunning.

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Action of Thiamine Applied Directly to the Cerebral Cortex

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It is generally claimed that vitamin B_1 (thiamine) exerts influence on the nervous functions. This assumption is based more on data obtained from deficiency states than on an effective action of thiamine on the specific functions of the nervous system. Indeed, it has been verified that this vitamin presents no typical or characteristic pharmacological effect on normal animals. Large doses by the intravenous route are tolerated, showing only discrete reactions in the blood pressure or urinary excretion (*7*, *12*). Death occurs by respiratory failure (*12*).

On the other hand, there is some experimental evidence indicating a possible association of thiamine with acetylcholine in the processes of nervous excitation. Binet and Minz (*3*) have

Voprosy Pitaniya 17(4): 48-53, 1958.

Toxicity Studies of Chemicals Added to Foods

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Until now, food toxicology has not found its theoretical and methodological determinations as a separate division of general toxicology, as a unique part of food hygiene.

Because of the increasing number of chemical food additives in circulation, it becomes necessary to work out unique methodological principles in the realm of food toxicology. In emphasizing this necessity we focused on specific problems arising during research on each separate substance.

Relative methods, currently in common use, have certain principal features, namely:

1. Modern research methods require much time and costly equipment, and consequently the possibility of conducting experiments is lower than existing losses.
2. Despite many existing indicators, none of these is general in character and removes the possibility of damage to the organism in sufficiently short a time to demonstrate aspects of substances clearly toxic. The necessity of such indicators was stressed by N. V. Lazarev at the All-Union Meeting of Hygienists in Leningrad in 1956.
3. There are no specific tests for determining the effects of specific substances.
4. The absence of standardized methods, which can produce false results, greatly complicated interpretation of results.

The indicated insufficiencies and especially the absense of specific methodical principles are the reason why certain toxicological studies on several substances give conflicting results and why the conclusions of these studies cannot be used without reservation. As eamples we cite the evidence of Roy and our group concerning the toxicity of ethyl gallate. In studying its effect on the growth of young rats, Roy did not observe changes when 1% ethylgallate was given in fat, while our observations recorded serious changes at 0.75% ethyl gallate. The differences in results can be explained by differences in investigational methods. It is necessary to stress that the truth of our conclusions were independently confirmed by other investigators, especially by the demonstration of histological changes in the animal's central nervous system(CNS).

In our studies lasting 4--5 years concerning the action on the animal organism of food preservatives(salicylic and benzoic acids) and also ethylgallate and antioxidant, substances that prolong the storage life of potatoes(methyl ester of alphanapthylacetic acid), dyes(orange GGN, amaranth, citrongelb), corn, protectors against Fusarium moniliforme, and wine stored in barrels (plywood) sealed with phenylformaldehyde resins, we strove to improve methods of toxicological investigation.

TABLE I

Growth of rats, maintained on a normal diet and on a diet with salicylic acid

Diet	initial in g.	Average weekly weight in g. I	II	III	IV	V	VI	VII	VIII	wt. at end of exper. in % of init.	weight in relation to initial w	
											g.	%
synthetic with 18% casein	40,1	55,6	67,5	80,8	103,3	122,7	140,6	160,6	166,8	415,9	126,7	315,9
same with 1 g% salicylic acid	39,6	58,4	76,8	97,0	118,6	134,6	149,6	159,4	168,2	424,7	128,6	324,7
" + 5 g% salicylic acid	42,2	58,5	72,6	87,6	101,3	114,3	125,5	134,3	138,8	328,9	96,6	228,9

TABLE 2

Weight gain in rats maintained on normal diet and on a diet with salicylic acid (average data in %)

diet	week I	II	III	IV	V	VI	VII	VIII	average for whole experiment
synthetic with 18% casein	39,5	29,7	32,6	60,0	49,7	45,9	53,9	15,4	40,8
ditto + 1g% salicylic acid	46,9	41,2	58,3	55,1	41,2	38,5	29,4	22,2	41,6
"+ 5g% salicylic acid	41,0	30,4	36,2	35,2	32,7	26,8	21,9	10,6	29,4

TABLE #3

Effect on growth of certain chemicals added to food products

substance studied	no. of animals	diet	concentration of substance	growth stimulation in weeks apparent, 1 *	real 2 **	
benzoic acid	12	casein, 18%	1 g%	11	3	3.7
"	12	"	5 g%	7	2	3.5
"	12	"	10 g%	3	1	3.0
Salicylic acid	6	"	1 g%	6	3	2.7
"	6	"	5 g%	4	3	1.3
ethyl gallate	8	"	0.75 g% in fat	3	2	1.5
"	8	casein 8%	"	3	3	1.0

*"Apparent" stimulation--weeks in which the average weight of the experimental animals was higher than that of the controls

**"Real" stimulation--weeks in which the average growth rhythm was higher in the expermental animals than in the controls

In the first place, in our experiments we used general methods employed by the majority of investigators in food toxicology regardless of the peculiarities of the substance under study, namely: a)in establishing the acute toxicity of the substance, b) its effect on the growth of young animals, c)its effect on the blood picture.

A)Establishing the acute toxicity of a substance. In methods used by various authors for the establishment of acute toxicity of a substance,we find great differences, which are as follows: various means of introducing the substance (per os, intraperitoneally, intravenously,subcutaneously etc.); different animal species (mice, rats, dogs, etc.);variations in the number of experimental animals,number of days in the experiment during which observatons were made etc.; and differences in the treatment of experimental results(LD_{min},LD_{max}) during single or repeated experiments and introduction of the substance; differences in the evaluation of results obtained(establishing simple curves,linear vectors etc.). From these differing results,obtained by various authors,it is impossible to compare and determine how,in aech variant ,they obtained their results. In our experiments, we used the following method to determine acute toxicity.

The substance was given to mice intraperitoneally in progressive doses; for a substance with marked toxicity we started at a dose of 100 mg/kg wt.(100,200,300 etc.), and for weakly toxic substances--from doses of 500 mg/kg (500,1000,1500 etc.)Animals were close together by weight,there were about 20 in the experimental group and these were observed seven days. To establish the LD_{50} we studied a linear curve at varying quantities of the administered substance.

Under the indicated conditions, theLD_{50} of which were higher than 1000mg/kg wt.,these substances were non toxic in chronic experimen In studying more toxic substances, were gave them per os to rats. Later we adopted another method: after establishing the LD_{50} of a substance given repeatedly (every 2 days), we gave it in doses leading to the death of all animals.
Using this method we attempted to detect the possible acute, continuous effect of the substance,the sensitization and adaptation of the animal to it.

Further methods, considered by some to be better,require unification for evaluation of the results obtained and for the establishment of comparative acute toxicity tables for various substances.

B. The Effect of the Studied Substances of the Growth of Young Animals.In our experiments, we used as a general indicator the weight of the animal--an indicator which at present is used in most food toxicology research, and we considered that it changes only under the influence of highly toxic substances or during the use of the substance in large doses. Thus, for example, in observing the effect of salicylic acid on the growth of young rats(in concentrations of 1 and 5 g%)we observed a weight loss only at the higher concentration;the weight of rats receiving 1 g% salicylic acid was close to that of the control group (168.2 g in comparison with 166.2 g). At the same time,in observing weekly growth rate(growth rhythm),we noted that in thefirst three weeks in animals receiving 1 g% salicylic acid, the average weekly growth rate increase was 44% higher than in the control group, and in the following 5 weeks--35% lower. These data suggest that the administration of salicylic acid in 1 g% concentrations depresses the growth rhythm from the fourth

week, despite the fact that the weight of the animals did not reflect this alteration. Such phasicity in action, that it the presence of a growth stimulant phase, which changes to a depression has been observed by us in testing several substances (tables 1,2,3). At the same time, that some substances stimulated growth (salicylic, benzoic acids, ethylgallate), other substances which appeared nontoxic in our experiments, did not have such phasicity (orange GGN, the methyl ester of alphanapthylacetic acid etc.).

Interest is shown in the fact that, in experiments with the food coloring orange GGN, in the experimental group of rats fed 18% casein, thegrowth stimulant phase was absent, while when the diet contained 8% casein (i.e. under conditions of incomplete nutrition) it was present. From analysis of the relation between "apparent" growth stimulation (the weight of the experimental animals compared with that of the controls) and the "real" stimulation (growth rhythm in comparison with controls) showed the the size of the ratio decreases with increasing toxicity of the substance. In experiments in which no growth stimulation was observed, this ratio could not be established.

It follows that, during the "apparent" stimulation period some of the animals receiving ethylgallate and a diet containing 8% and 18% casein, the ratio between "apparent" and "real" growth stimulation was lower in animals receiving a diet with 8% casein, which corresponds with general evaluation of the toxicity of the substance under the given conditions. We consider that our data is insufficient to give final conclusions about the problems connected with the phasic action of chemical food additives, but the solution of the problem is of great interest.

Presently one can made a tentative conclusion that in studying the effect of chemicals on the growth of animals, one must consider not only their weight, but their weekly weight increase, that is the growth rhythm. Our experiments showed, however, thatin the absense of a weight and growth effect by these substances, there can be different effects on the animals. Thus, for example, in studying the effect of the food dye amaranth on rats fed a diet with 20% and 11% casein, the amount of food consumed was 12% higher in the experimental group than in the control group, that is, there was poorer utilization of food for growth. Evidently, along with data on growth and weekly weight increase, one must consider the quantity of ingested food to establish indicators of protein effectiveness (weight increase in grams per gram of protein eaten.).

In considering the protein effectiveness indicator, we have the possibility of following the effectiveness of the indicated substance not only on growth, but also on protein utilization for growth. To illustrate this theory, we compared results, obtained by using protein effectiveness indicators, with results obtained by direct measurement of protein utilization, namely, by a method of regenerating liver proteins after starvation.

The resultsof studying four groups of animals fed salicylic and benzoic acids show that the protein effectiveness indicator directly reflects the degree of protein metabolism inhibition under the influence of the given factor.

Since our experiments with animals involved complete utilization of food, the question arose of studying the effect of the indicated substances on animals maintained on a low protein diet (8--12%) that is, under conditions of some nutritional deficiency. The results of our studies concerning the toxicity of ethylgallate show that ,in a diet with 8% casein the changes in the animal organism are 50% more than those appearing with and 18% casein diet. This shows that the use of low protein diets in growth studies is a very sensitive method. Simultaneously it is stressed that the use of diets with varying protein concentrations (low and optimum) serves to evaluate the degree of influence exerted by a given substance on protein metabolism.

In carrying out such experiments, it is necessary to observe the following conditions: use animals of one litter(rats), one age(20--27 days), one sex,similar weights (about 30 g) and no less than 8 in each experimental group.The substance should be given per os,either pipetted or in drinking water. At the time of the experiment the animals should be placed in individual cages and fed an unlimited amount of synthetic ration of the following composition: casein 8 or 18%,starch 64 or 74%, sunflower oil 8%,dry yeast 4%, Osborne-Mendel salts mixture 4%,agar 2%. Additionally,each week in the oil, animals should be given 20--30 IU per os vitamin A and D. Food and waste should be weighed daily and the animals, every 6 days.

Standardization of the experiment is very important. We will speak on that from our own observations in which the substitution of one feed for another with the same nutrient composition led to a definite change in the amount of food utilized--adversely,which changes appeared in about a week.

The use in our experiments of growth tests conducted under the indicated conditions led to convergant results in the experimental group; the difference in average weight of the group did not exceed 1--2 g.

Finally, we did not obtain such convergent results in all experiments. Thus, for example, in the study of weakly toxic substances growth changes occurred which were possible to evaluate only after statistical treatment of the material. As an example we refer to our data on the toxicity of corn contaminated with Fusarium moniliforme. Thus, the average weight of the rats at the end of the experiment reached 223.2 ±7.57 g in the control group and was 209±7.65 g in the experimental group, that is, the difference was 14. 2 g. Statistical treatment of the results using Student-Fischer"s reliability criteria showed that the difference was statistically inadequate: t=0.87 lower than 2.101.

In case of dubiuos experimental results the identification and establishment of other factors is required.

It is unarguable that growths tests are not the only indicator of the total animal response--by such an indicator we can follow for example,changes in the higher nervous activity,but this test is advantageous because it is simple, does not require complex equipment, can use a large number of animals and yields statistically treatable results. Additionally,it is applicable to subacute and chronic experiments.

The indicated use of growth tests and our experiments in testing the effects of some food additives added in measured quantities at small concentrations is used in industry(preservatives, dyes, antioxidants etc.).Under these conditions synthetic feed may be used in the tests.

In studying the toxicity of trace amounts of chemicals in foods (insecticides, fungicides etc.) the use of growth tests under these conditions is difficult and there is some trouble in evaluating food intake, protein utilization effectiveness etc.

Thus our experiments in this field were limited and we do not consider further investigations of growth tests in this area to be fruitful.

C.)<u>The Blood Picture</u>. The study of the blood picture is widely used in food toxicology as a general and specific indicator. The difficulty connected with the use of this method involves chiefly the analysis of results, since the studied substance often cannot produce visible change. Such changes are often caused, not by the studied substance,but by individualized variations in the blood picture of the experimental animals.

In studying the blood picture of 81 healthy rats(243 analyses) we observed more variation in the number of leucocytes per mm^3 (on the average 6030--12,104; as a consequence of which, the normal average variation exceeded statistically reliable quantities,reaching in one group ± 2794 at an average quantity of 11,360. The individual variations in hemoglobin and erythrocyte numbers did not exceed statistically reliable limits. These data indicate that, in evaluation of results concerning the effect of a given substance on leucocyte number,one must proceed extremely carefully. In our experiments we considered only those changes which were confirmed by three analyses at three week intervals and which were statistically reliable.

In addition to the methods discussed, we also used the method of liver protein regeneration after fasting, sugar stress,investigations of carcinogenic effect etc.Our accumulated material concerning these factors is insufficient, and therefore their analysis will be the subject of our future research.

J. Am. Chem. Soc. 76: 2848, 1954.

PHENYLACETYLGLUTAMINE AS A CONSTITUENT OF NORMAL HUMAN URINE

Sir:

Phenylacetylglutamine (PAG) was first described by Thierfelder and Sherwin[1] who isolated the compound from the urine of individuals fed phenylacetic acid. The conjugate is frequently referred to as a "detoxication" product. In investigating the source of the amino acids liberated by acid hydrolysis of human urine,[2] it has been found that PAG is excreted under normal conditions by the adult male to the extent of 250 to 500 mg. per day, and accounts for about 50% of the conjugated glutamic acid in urine.

Identification of PAG has been effected by chromatographic analyses of urine employing acidic and basic ion-exchange resins (Dowex 50 and Dowex 2). PAG and other conjugates were detected in the effluent by hydrolysis of 1-ml. effluent fractions with 6 *N* HCl or 2 *N* NaOH prior to the application of the photometric ninhydrin method.[3] Because of the affinity of the resin for aromatic compounds, PAG, despite its acidic nature, is retarded on columns of Dowex 50X8 and emerges at a position between urea and aspartic acid.[2] The conjugate was more readily determined by chromatography of 4-ml. samples of urine on 0.9 × 30 cm. columns of Dowex 2X4 (200–400 mesh) in the acetate form. Elution was begun with 0.2 *M* sodium acetate buffer at *p*H 5.3.[4] After 100 ml., the *p*H and ionic strength were gradually changed by allowing a 2 *M* sodium acetate buffer of *p*H 4.6[5] to flow into 100 ml. of the initial 0.2 *M* buffer stirred magnetically. The compound giving rise to a major peak at about 195 ml. was identified as PAG by the demonstration that the unknown and a sample of synthetic PAG[6] exhibited identical chromatographic behavior on columns of both Dowex 2 and Dowex 50, together with the finding of equimolar amounts of glutamic acid and ammonia in an acid hydrolysate of the unknown.

The same analytical procedure afforded a chromatographic determination of hippuric acid, which emerged at about 245 ml. from the Dowex 2 column. Normal adult males may excrete 1.0 to 2.5 g. of hippuric acid per day, a value which is higher than others previously reported and accounts for 65 to 75% of the observed conjugated glycine in 24 hour urines.[2] Hippuric acid and PAG together account for about half of the 2 g. of bound amino acids excreted daily.

The quantities of PAG and hippuric acid observed in the urines from fasting individuals, were similar to those reported above, indicating that both compounds probably are normal metabolic products, and do not arise only as a result of the "detoxication" of dietary precursors. In phenylpyruvic oligophrenia, PAG excretion was 2.4 g. per day (*cf.* 7) whereas the quantity of hippuric acid was diminished to about 0.3 g. In Wilson's disease, urinary hippuric acid was similarly reduced in amount but PAG excretion was normal.

We wish to acknowledge the technical assistance of Miss Joyce F. Scheer.

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(4) 27.2 g. of NaOAc·3H₂O + 5.0 ml. of glacial HOAc + 2.5 ml. of 50% BRIJ-35 solution diluted to 1 l. with water. BRIJ-35 is a detergent manufactured by Atlas Powder Co., Wilmington, Delaware.

(5) 54.4 g. of NaOAc·3 H₂O and 10.0 ml. of glacial HOAc are made to a volume of 100 ml. with water. To this solution 30 ml. of glacial HOAc, 70 ml. of water and 0.2 ml. of BRIJ-35 solution are added.

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J. Biol. Chem. 62: 565-573, 1925.

THE EFFECT OF SODIUM BENZOATE INGESTION UPON THE COMPOSITION OF THE BLOOD AND URINE WITH ESPECIAL REFERENCE TO THE POSSIBLE SYNTHESIS OF GLYCINE IN THE BODY.

PRELIMINARY PAPER.

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(Received for publication, June 26, 1924.)

Since the work of Wiechowski (1) in 1906 several investigators have confirmed the fact that the excretion of urea is decreased after ingestion of sodium benzoate. In 1916 Lewis and Karr (2) found that uric acid was also markedly decreased in the urine under similar conditions. From these findings it was suggested that, where there is an abnormal demand for glycine, this amino acid is synthesized, in part, from the end-products of protein metabolism.

Recent experiments by Gibson and Doisy (3) indicate that some organic acids such as lactic and benzoic have the tendency to decrease the excretion of uric acid and to cause a slight increase of the same in the blood. However, figures showing the effect of the ingestion of sodium benzoate were not included in their paper.

It is the aim in this paper to present values for the non-protein nitrogen constituents in the blood and urine obtained by simultaneous analyses after ingestion of sodium benzoate; further, to investigate what bearing these values may have, if any, on the synthetic ability of the human body to produce glycine for conjugation with the benzoic acid.

Analytical Methods.

All the experiments were done on young healthy individuals. The blood was drawn from an arm vein and immediately transferred to a test-tube containing 20 mg. of potassium oxalate per

10 cc. of blood to prevent clotting. Filtrates were always prepared a few hours after being drawn and in every instance analysis of the blood was completed during the ensuing 24 hours. Drawn blood as well as filtrates were always kept in the refrigerator to minimize changes in composition. The preparation of protein-free blood filtrates and determination of total non-protein nitrogen, urea, and sugar were done according to directions found in Folin's Manual (4). Duplicate analyses were always made and, if these did not check, a second determination was completed. The uric acid in the blood was determined by the new method of Benedict (5). The amino acid nitrogen determination was carried out on the same filtrates using Folin's (6) colorimetric method, and the plasma proteins by the colorimetric method of Wu (7).

In order that the data presented should be more complete, hourly specimens of urine were taken during the experimental period. A 4 hour morning specimen was generally used as control. The urines were preserved by adding 2 cc. of a 2 per cent nitric acid solution and a few drops of a 10 per cent solution of thymol-chloroform.

The amino acid nitrogen determination in urine was made as described by Folin (8) on hourly specimens. The determination of hippuric acid was carried out by the method of Kingsbury and the author (9), uric acid by the method of Benedict and Franke (10), and urea nitrogen by the method described in Folin's Manual (4).

To insure uniformity and obviate the influence of food, a slice of toast and a cup of coffee were taken in the morning, but no further food during the time of the experiment. There was no restriction placed upon the water intake, and in none of the experiments did the intake exceed the ordinary amount for the individual.

EXPERIMENTAL.

Tables I and II are submitted as controls for the work to follow. The constituents listed for blood and urine are well within the normal range when compared with values reported in the literature.

In Tables III, IV, and V are shown the values for blood and urine after ingestion of 3 to 4 gm. of sodium benzoate. Some

TABLE I.*

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.					Plasma.				
	Amino acid N.	Urea N.	Total non-protein N.	Uric acid.	Sugar.	Amino acid N.	Urea N.	Total non-protein N.	Uric acid.	Sugar.
p.m.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
12.20	8.3	17.0	37	3.0	90	6.4	16.6	23	3.6	97
6.15	7.0	16.6	33	3.0	83	6.0	16.6	22	3.4	91

* Average of a series of five experiments.

TABLE II.*

Subject, W. W. S. Weight, 76 kilos.

Analysis on 1 hr. specimens of urine.

Time.	Volume per hr.	Amino acid N.
a.m.	cc.	mg.
6.40 to 11.30	54	4.2
p.m.		
12.30	58	4.7
1.40	60	3.7
3.30	38	3.2
4.30	36	3.2

* Average of a series of three experiments.

TABLE III.

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.			Plasma.			Urine per hr.			
	Amino acid N.	Urea N.	Total non-protein N.	Amino acid N.	Urea N.	Total non-protein N.	Volume per hr.	Amino acid N.	Hippuric acid.	Glycine.
a.m.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	cc.	mg.	gm.	gm.
7 20 to 11.00*	6.6	19	33	5.5	16.7	30	46	6.7	0.084	0.035
p.m.										
12.00	6.4	14	30	5.5	15.0	27	71	5.9	0.994	0.44
1.00	6.4	15	32	5.9	13.0	28	253	5.1	1.85	0.77
2.00	5.7	15	30	5.9	14.0	30	77	3.9	0.73	0.31
4 30	5.7	15	30	6.2	15.0	27	46	3.5	0.18	0.07

* Took 3.34 gm. of sodium benzoate.

TABLE IV.

Subject, H. H. J. Weight, 86 kilos.

Time.	Whole blood.			Plasma.		Urine per hr.			
	Amino acid N.	Urea N.	Total non-protein N.	Amino acid N.	Total non-protein N.	Volume per hr.	Amino acid N.	Hippuric acid.	Glycine.
a.m.	mg. per 100 cc.	mg. per 100 cc	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	cc.	mg.	gm.	gm.
11.30*	9.0	13.0	30	7.3	35	60	6.0		
p.m.									
12.30	7.9	14.4	41	7.0	34	140	5.4	1.56	0.652
1.30	8.5	11.4	41	7.6	35	405	6.3	1.89	0.794
2.30						88	4.5	0.62	0.262
3.30	8.2	13.0	40	8.4		50	5.0		
5.00						26	3.8		

* Took 3.65 gm. of sodium benzoate.

TABLE V.

Subject, B. B. Weight, 76 kilos.

Time.	Whole blood.			Urine per hr.			
	Amino acid N.	Urea N.	Total non-protein N.	Volume per hr.	Amino acid N.	Hippuric acid.	Glycine.
a.m.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	cc.	mg.	gm.	gm.
6.40 to 11.15*	11.0	12.5	36	86	8.2		
p.m.							
12.15	8.3	15.7	34	106	6.3	1.31	0.553
1.15				95	4.8	1.57	0.658
1.20	8.7	12.0	31				
3.15	8.7	16.0	32	41	3.4	1.05	0.440

* Took 3.5 gm. of sodium benzoate.

TABLE VI.

Subject, A. G. M. Weight, 65 kilos.

Time.	Whole blood.			Plasma proteins.		
	Amino acid N.	Urea N.	Total non-protein N.	Fibrin.	Globulin.	Albumin.
p.m.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent	per cent	per cent
12.20*	8.2	16.6	48.4	0.22	2.1	5.2
2.10	7.4	10.8	31.2	0.23	2.0	5.1
3.30	9.3	12.5	43.2	0.22	2.0	5.2

*Took 3.30 gm. of sodium benzoate.

variability appears to exist in the values obtained for the different individuals. These variations are, however, too small to draw any definite conclusions from, but may indicate to some extent the effect of the benzoate upon normal values in blood and urine.

TABLE VII.

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.			Plasma.			Plasma proteins.		
	Amino acid N.	Urea N.	Total non-protein N.	Amino acid N.	Urea N.	Total non-protein N.	Fibrin.	Globulin.	Albumin.
p.m.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent	per cent	per cent
12.00*	7.2	23.0	46	6.4	19.4	28.4	0.32	2.2	4.7
2.00*	7.0	17.5	42	4.5	18.7	26.0	0.33	2.3	4.4
4.00	5.9	16.5	34	4.9	18.0	25.0	0.33	2.3	4.4
6.00	5.5	17.6	34	5.0	20.0	25.0	0.34	2.3	4.7

Urine per hr.

Time.	Volume per hr.	Amino acid N.	Urea N.	Uric acid.	Hippuric acid.	Glycine.
a.m.	cc.	mg.	mg.	mg.	gm.	gm.
7.00 to 11.50	60	7.5	540	62		
p.m.						
12.00*						
12.50	198	7.2	166	13	1.03	0.43
1.50	215	5.5	150	11	1.25	0.52
2.00*						
2.50	170	4.9	136	8	1.44	0.60
3.50	172	5.3	136	7	1.56	0.65
5.00	134	4.6	134	9	2.86	1.20
6.30	90	4.3	135	15	1.13	0.47

* At 12.00 n. took 3.35 gm. and at 2.00 p.m., 6.70 gm. of sodium benzoate.

In Tables VI and VII is shown the stability of plasma proteins under varying quantities of ingested sodium benzoate. The blood values in Table VII with a trebled dose of sodium benzoate differ in no particular from those listed in Tables III, IV, and V.

A more complete analysis of the urine in Table VII confirms again the established facts that urea and uric acid are markedly decreased after ingestion of benzoate.

TABLE VIII.

Subject, A. G. M. Weight, 65 kilos.

Time.	Whole blood.				Plasma.			
	Urea N.	Uric acid.	Total non-protein N.	Sugar.	Urea N.	Uric acid.	Total non-protein N.	Sugar.
p.m.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
12.40*	19	4.2	34	98	19	4.8	26	130
2.40	19	4.8	33	90	20	5.3	23	118
4.40	19	4.8	42	83	20	5.5	29	100

Urine per hr.

Time.	Volume per hr.	Urea N.	Uric acid.
	cc.	mg.	mg.
8.00 a.m. to 12.40 p.m.*	40	420	28
p.m.			
1.40	51	216	9
2.40	65	140	8
3.40	163	195	9
4.55	40	150	12

* Took 5 gm. of sodium benzoate.

TABLE IX.

Subject, W. W. S. Weight, 76 kilos.

Time.	Whole blood.			Plasma.		
	Urea N.	Uric acid.	Total non-protein N.	Urea N.	Uric acid.	Total non-protein N.
p.m.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
12.40*	19.0	3.6	33.0	17.0	4.2	25
2.40	15.4	4.0	31.0	16.8	4.5	22
4.40	15.0	4.0	36.0	16.0	4.5	22
5.40	14.0	4.4	30.0			

Urine per hr.

Time.	Volume per hr.	Urea N.	Uric acid.
	cc.	mg.	mg.
7.30 a.m., to 12.40 p.m.*	35	580	24
p.m.			
1.45	90	332	6
2.50	85	280	4
3.50	200	380	7
4.50	345	345	9
5.50	66	228	14

* Took 10 gm. of sodium benzoate.

To show just what happens to the blood uric acid Tables VIII, IX, X, and XI are submitted. There is a definite rise in the uric acid content of both the whole blood and plasma, while the urine analyzed simultaneously shows the marked decrease.

TABLE X.

Subject, Wm. K. Weight, 70 kilos.

Time.	Uric acid.		Urine per hr.	
	Whole blood.	Plasma.	Volume per hr.	Uric acid.
a.m.	*mg. per 100 cc.*	*mg. per 100 cc.*	*cc.*	*mg.*
7.15 to 8.15			22	18
8.00*	4.3	5.3		
8.35	4.3	5.3		
10.45			52	3
p.m.				
1.30	4.3	5.3	52	3
3.30	4.7	5.7	37	4

* Took 10 gm. of sodium benzoate.

TABLE XI.

Subject, Wm. K. Weight, 70 kilos.

Time.	Uric acid.		Urine per hr.	
	Whole blood.	Plasma.	Volume per hr.	Uric acid.
a.m.	*mg. per 100 cc.*	*mg. per 100 cc.*	*cc.*	*mg.*
7.00 to 8.00			24	18.0
8.30*	4.0	5.0		
10.30			60	2.7
12.00	4.2	5.3	52	2.0
p.m.				
2.30	4.7	5.2	33	2.5

* Took 10 gm. of sodium benzoate.

DISCUSSION.

From the experimental findings there may be some support for the supposition that the constituents, which during ordinary metabolism enter into the composition of urea, may in part be synthesized into glycine. If it were merely a retardation of excretion, then there should be an increase of urea nitrogen in the blood. This, however, is not the condition, for in most experiments the blood urea nitrogen is slightly decreased.

The values obtained for uric acid in blood lead to a different conclusion. While the experiments are few in number, there appears to be a gradual increase in uric acid in the blood and plasma after benzoate ingestion. This fact seems to contradict the suggestion of Lewis and Karr (2) who thought that a decrease of the uric acid in the urine might mean a possible hydrolysis of this acid and synthesis of the resulting products into glycine. It is more probable that the excretion of uric acid is slightly retarded whenever large amounts of hippuric acid are being eliminated.

It was hoped that a study of the blood and urine amino acid content, after sodium benzoate ingestion might throw some light on the nature of the circulating amino acids; that is, if the total amount of these was moderately lessened at the time of rapid hippuric acid formation, it would be reasonable to believe that the circulating amino acids were largely composed of glycine. Experiments recorded in Tables III, IV, and VII, showing little or no appreciable difference in the plasma amino acid after relatively large doses of sodium benzoate, indicate that this is not the case. The urine amino acid per hour shows a decrease after benzoate ingestion in all the experiments; but this is also noted in the experiment shown in Table II in which no benzoate had been taken and is due merely to inanition.

SUMMARY AND CONCLUSION.

After ingestion of sodium benzoate the simultaneous analyses of blood and urine of normal individuals show: (1) no increase in the urea nitrogen content of the whole blood and plasma, but a decrease in the urea nitrogen of the urine; (2) an increase of the uric acid content of the whole blood and plasma, and a marked decrease in the uric acid content of the urine; (3) the glycine portion of the blood amino acids and of those which are normally excreted while fasting appears to be only a small percentage, if any, of the total; and (4) the experimental findings suggest a probable synthesis of glycine from the constituents which normally are converted into urea, but it appears improbable that uric acid supplies any glycine.

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The Journal of Biochemistry, Vol. 41, No. 3, 1954

INTERRELATION BETWEEN THE FUNCTION OF HEME-PROTEINS AND THE STRUCTURAL MODIFICATIONS OF THEIR PROTEIN PARTS

II. EFFECT OF BENZOATE UPON THE AUTOXIDATION OF OXYHEMOGLOBIN

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(Received for publication, December 22, 1953)

The essential function of hemoglobin as an oxygen transporting system can easily be affected even through a mild structural alteration of its globin part, for instance, through a reversible denaturation caused by acids, by pyridine or by addition of Cu^{++}. Interestingly enough, this is accompanied by an alteration of its catalytic function, the modified hemoglobin thus becomes significantly easily autoxidizable as pointed out by Holden (*1*, *2*), Rawlinson (*3*) and Lemberg (*4*).

In his previous paper, the present author reported on the spectral changes of hemoglobin and hemiglobin induced by the addition of benzoate. These spectral changes were suggested by the author to be the result of the coordinative linking of heme-Fe to another globin-N group which was opened out from the globin molecule through its configurational modification induced by benzoate. From his interest on the interrelation between the structure of hemoglobin and its catalytic function, the present author intended to study the possible alteration of the catalytic function of oxyhemoglobin according to the structural modification of its globin part by benzoate.

EXPERIMENTAL METHODS

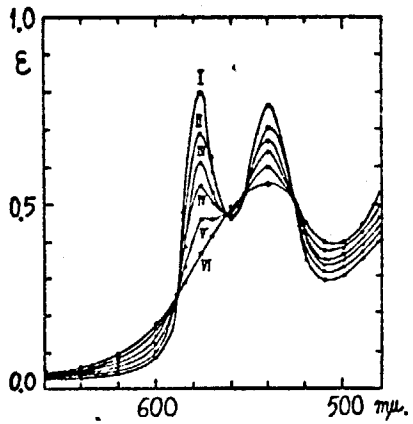
Bovine erythrocytes repeatedly washed by physiological saline solution were laked by addition of distilled water and centrifuged. The supernatant was used as the oxyhemoglobin solution for the present experiments. All the blood samples were reserved in ice box and used for the experiments within 24 hours. Concentrations of oxyhemoglobin were checked at each experiment by the determination of its optical density at 578 mμ. 2 *M* solution of benzoate was prepared as the stock solution, from which the solutions of desired concentrations were made each time by dilution.

Each of the solutions of oxyhemoglobin and benzoate was separately pipetted into

beakers of 200 ml. content and incubated at 30°. Two solutions were then mixed after they reached the constant temperature. 9.5 ml. of the reaction solution was pipetted out from the beaker at certain intervals of time, 0.5 ml. of 1.0 per cent KCN solution was added and the spectrophotometric reading was made within 1 minute. pH of the solution was determined by means of glass electrode. Temperature of the experiments was kept constant at 30°, except those of the temperature change. The optical determinations were carried out by the photoelectric spectrophotometer of Hitachi Company.

RESULTS AND INTERPRETATIONS

I. Determination of the Reaction Rate by Absorptiometrical Observations— To the solution of oxyhemoglobin, benzoate was added to its final concentration of 1.785 *M*. Optical density was measured after 30 minutes of incubation at 30°. An absorption figure thus recorded was perfectly similar to that obtained from the solution of methemoglobin after the reaction with benzoate (*5*) *i.e.* a typical hemichrome figure. Thus, for the present reaction process, two possible modes of reaction may be taken into consideration, one of which being the way to hemichrome from oxyhemoglobin directly and the other being the way to hemichrome stepwise from oxyhemoglobin through methemoglobin as an intermediate. When, however, KCN was added to this hemichrome like compound, an absorption figure was obtained which was similar to CN-methemoglobin. The process of the spectral change of oxyhemoglobin into hemichrome was thus followed up, for convenience, by the CN-methemoglobin spectrum formed on addition of KCN into the reaction solution. The problem regarding the reaction modes will be discussed in a later section. To the solution of oxyhemoglobin, benzoate was added to its final concentration of 1.052 *M* and after certain intervals of time, KCN was added to it. The resulted absorption figure was thus recorded as in Fig. 1. The absorption maxima at 578 and 540 mμ decreased with time until finally the figure of CN-methemoglobin was formed. The curves in Fig. 1 make four isosbestic points indicating the reaction system of two components: oxyhemoglobin and CN-methemoglobin, the latter being formed from hemichrome by KCN added. The percentage of oxyhemoglobin autoxidized in the system could then be obtained from the value of ε_{578} by a nomogram established by a procedure similar to that described in the previous paper (*5*). Now the percentages of autoxidation were determined at varied concentrations of benzoate and after each time interval. The plotts of log percentages of remaining oxyhemoglobin against time were recorded in Fig. 2. The relation was



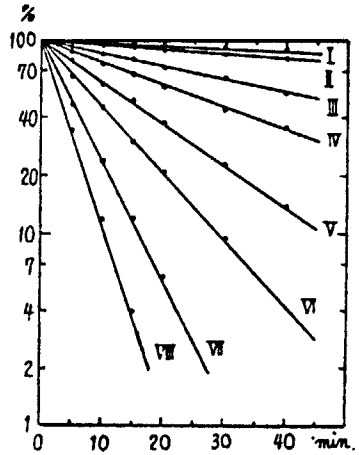


FIG. 1. The absorption change on addition of benzoate to oxyhemoglobin solution.

On measurements, KCN solution was added, so that the final figure recorded corresponds to that of CN-methemoglobin. Final concentration of benzoate was 1.052 *M*. The readings were made after the following intervals of time.

I ; 0 min. II ; 10 min. III ; 20 min. IV ; 30 min. V ; 50 min. VI ; 120 min. pH=7.2 T=30°

FIG. 2. The plotts of log percentages of remaining oxyhemoglobin against time.

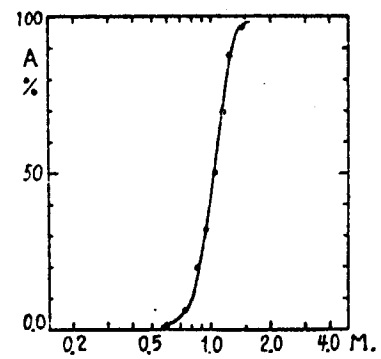
Concentration of oxyhemoglobin ; 0.085% Concentration of benzoate ; I ; 0.61 *M*, II ; 0.74 *M*, III ; 0.86 *M*, IV ; 0.965 *M*, V ; 1.052 *M*, VI ; 1.17 *M*, VII ; 1.26 *M*, VIII ; 1.42 *M*, pH ; 7.2, T=30°

given as a straight line at each of the benzoate concentrations, indicating a first order reaction in respect to the concentration of oxyhemoglobin. Now, taking V_O as the reaction velocity without benzoate and V_B as that with benzoate, the percentage of increase in the reaction velocity (A) promoted by benzoate can then be expressed by $A=(1-\frac{V_B}{V_O})\times 100$. From experiments with benzoate in varied concentrations, the percentages of remaining oxyhemoglobin after 15 minutes were determined. By introducing these values into V_O and V_B of the above equation, the value

of A was calculated. As shown in Fig. 3, the plotts of A against log concentrations of benzoate gave a sigmoid curve. The value of n was calculated from the curve to be about 4.5. From this value, it may be assumed that the autoxidation of oxyhemoglobin seems to begin with the combination of globin molecule with 4 to 5 molecules of benzoate. Clarification of the actual feature of the combination between these two components, must await further experimentation.

II. Effect of Temperature—If the reaction by benzoate described above is to be assumed as the result of perturbation occuring in the molecule of protein part, it may be expected that the reaction can greatly be affected by temperature. The following experiments were undertaken, therefore, to investigate this point.

As shown in Fig. 4, the reaction rate increased with increase of



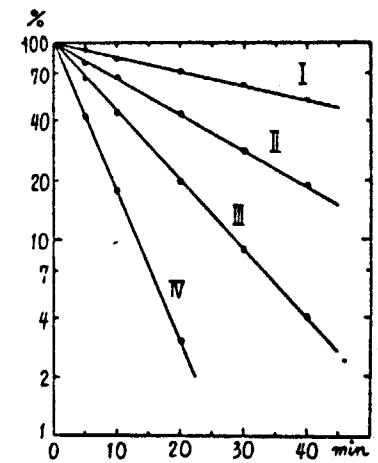


FIG. 3. The plotts of A (percentage of velocity increase) against log concentrations of benzoate.

FIG. 4. The effect of temperature upon the reaction velocity.

Concentration of oxyhemoglobin ; 0.085% Concentration of benzoate ; 1.30 *M*. pH ; 7.2 Temperature ; I 16° ; II 22° ; III 26° ; IV 30°

temperature, the final concentration of benzoate being kept constant at 1.58 *M*. The rate constants k were thus obtained at each temperature. The plotts of log k against the reciprocals of absolute temperature

revealed a linear relationship between them (Fig. 5). From the slope of the line the activation energy of Arrhenius was calculated to be 28.7 Cal./mole. The energy value thus obtained should be understood as the sum consisting of the energy of the combination of globin and benzoate, that of the structural change within protein molecule and that of the activation of molecular oxygen. Though the energy value obtained thus cannot be exclusively accounted for by the reaction of combination step of globin and benzoate, it is very much smaller than those so far reported for the reaction of protein denaturation, for instance, for the reactions of heat denaturation or alcohol denaturation of oxyhemoglobin (*6, 7*). It may then be assumed, at least, that the affection of benzoate upon oxyhemoglobin (by which the latter becomes easily autoxidizable) may be far from a sort of denaturation in the classical meaning but a slight structural modification of its globin part.

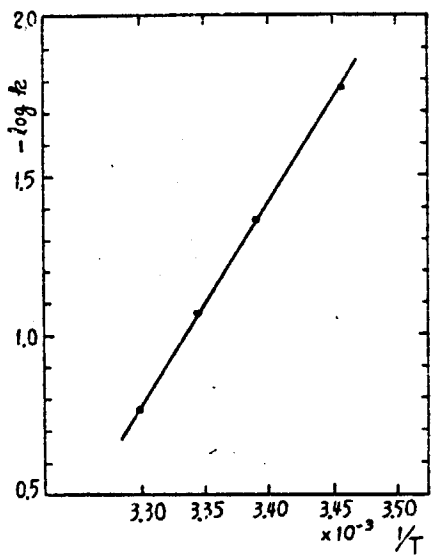


Fig. 5. The plotts of log k against the reciprocals of absolute temperature.

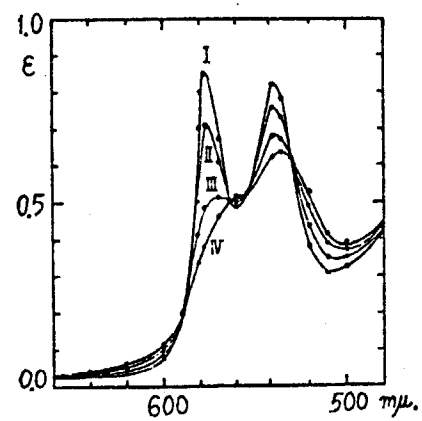
III. Reaction Process of the Autoxidation—Two possible elucidations for the above mentioned reaction process present themselves:

a) If the autoxidation of oxyhemoglobin occurs only after its protein part was so much affected by benzoate that the resulting heme-protein gives a hemichrome figure in its Fe^{+++} state, then the presented reaction process may correspond simply to a conversion of oxyhemoglobin to hemichrome;

b) but, if the structural modification of oxyhemoglobin necessary for inducing its autoxidation is slighter, then an intermediary formation of methemoglobin may be suggested and the latter may immediately turn into hemichrome. In order to explain the presented reaction, the following experiments were undertaken. Benzoate was added to oxyhemoglobin solution and the absorption changes thus occured were followed up at certain time intervals. Results are shown in Fig. 6.

including isosbestic points in the curves. From this the possibility of intermediary formation of methemoglobin can apparently be excluded. At this benzoate concentration, however, the conversion from methemoglobin to hemichrome took place almost instantly as reported in the previous paper, so that the intermediary methemoglobin, even though it might have been actually formed, could not be detected with certainty. The problem remains as yet a matter of conjecture.

IV. Accessibility of Several Heme-proteins to Benzoate—From the results of the present experiments including that of the spectral changes reported in the previous paper, the accessibility of several proteins to benzoate are summarized in Fig. 7. As may be seen from this figure, the



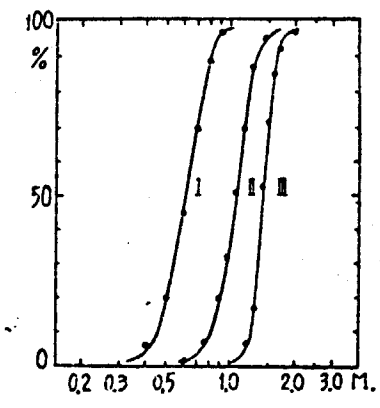


Fig. 6. The absorption change of oxyhemoglobin to hemichrome by the addition of benzoate to its final concentration of 1.052 *M*.

The resulted absorption figures were obtained at the following intervals of time: I 0 min.; II 10 min.; III 45 min.; IV 120 min.; pH 7.2 T=30°.

Fig. 7. The accessibility of methemoglobin, oxyhemoglobin and reduced hemoglobin to benzoate. (Summarized from the experimental results described above and reported in the previous paper).

I: from the absorption change of methemoglobin to hemichrome,
II: from the autoxidation velocity of oxyhemoglobin,
III: from the absorption change of reduced hemoglobin to hemochrome.

protein parts of heme-proteins, methemoglobin, oxyhemoglobin and

reduced hemoglobin, were affected by benzoate in progressively higher concentrations, in the given order. The accessibility of globin molecule to benzoate seems to be dependent on its molecular state in each heme-protein. An analogous assumption was afforded by Ross and Turner (*8*), who have found that the stability of home-proteins against tryptic digestion was greater in the order of methemoglobin, oxyhemoglobin and reduced hemoglobin. Both findings might possibly be explained on the basis of some structural reasons in common.

On the other hand, it was suggested by Perutz *et al.* (*9*) from their X-ray crystallographic studies, that the globin part in the oxyhemoglobin molecule is similar to that in the molecule of methemoglobin, and that it differs from that in reduced hemoglobin. Further, Wyman (*10*) suggested the structural similarity of oxyhemoglobin to methemoglobin on the basis of experimental results indicating that the Bohr effect observed in the oxidative conversion of hemoglobin to methemoglobin is essentially the same as that found in the oxygenation of hemoglobin and also in connection with the results of X-ray study by Perutz *et al.* (*9*).

The concentration of benzoate that is effective to induce the autoxidation of oxyhemoglobin was found in the present experiment, as described above, to be much higher than that effective to induce the spectral conversion of methemoglobin into hemichrome. From these findings connected with the above mentioned assumption of Wyman (*10*), it would not be unreasonable at present to assume that the protein part of oxyhemoglobin may have been more stabilized through its combination with O_2 than that of methemoglobin. The suggestion remains as yet to be proved with evidence; how far the autoxidation of oxyhemoglobin including the process of O_2 activation can be connected with the structural modification of globin in the chemical meaning as well as in the grade of the change remains so far unknown. The structural similarity between methemoglobin and oxyhemoglobin was proposed by Wyman (*10*) on the experimental basis of Bohr effect. But, how far the Bohr effect is in accord with the structural state of the globin part remains also to be investigated. From the assumption that reduced hemoglobin is in random structure, as suggested by Wyman (*10*), and from the fact that reduced hemoglobin can be affected only by higher benzoate concentrations than methemoglobin, it may further be suggested that the stability of heme-proteins against perturbators is highly dependent on the physical state of heme part in each of their molecules.

SUMMARY

1. Benzoate accelerated the autoxidation of oxyhemoglobin.
2. The autoxidation of oxyhemoglobin in the presence of benzoate proceeded according to the first order kinetics in respect to the concentration of oxyhemoglobin.
3. The relation between the percentage promotion of the autoxidation of oxyhemoglobin and the log concentration of benzoate could be expressed by a sigmoid curve of a high order reaction.
4. Activation energy in the autoxidation of oxyhemoglobin induced by benzoate was calculated to be 28.7 Cal./mole.
5. The stability of globin molecule against the action of benzoate differs with the difference of the physical state of their heme part.

This study was undertaken at the suggestion by Prof. K. Kaziro to whom the authors deep gratitude is due. I should like to express my thanks also to Dr. G. Kikuchi for his helpful discussion during the course of this work.

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Am. J. Med. Sci. 217: 563-568, 1949.

THE EFFECT OF BENZOIC ACID AND CARONAMIDE ON BLOOD PENICILLIN LEVELS AND ON RENAL FUNCTION*

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Since 1943, various substances which will interfere with the tubular excretion of penicillin have been studied. Diodrast[2,12,13] and para-amino hippuric acid[4,11] have proven to be effective but must be given by continuous intravenous administration in large doses and are not clinically practical for this reason. Benzoic acid[8,9,15] sodium benzoate, and more recently caronamide†[3,5,6] have been used orally. With the exception of caronamide all are thought to act by blocking the tubular excretion of penicillin. Bronfenbrenner and Favour[9] reported 4 to 7-fold enhancement of blood penicillin levels when benzoic acid was given orally but found that severe fluid and salt restriction was necessary to accomplish this. Spaulding, Bondi, and Early[15] also reported favorably on the use of this substance for enhancing blood levels of orally administered penicillin. Boger and Baker[7] have recently reported failure to increase blood penicillin levels by the use of benzoic acid when fluid and salt intake was not restricted.

In the last few months, several papers have appeared which deal with the clinical use of caronamide.[7,10] All have reported the effectiveness of this substance in increasing blood penicillin levels. Rather exhaustive renal studies in dogs have been carried out by Beyer,[3] who has been unable to find any evidence of renal damage from this drug. Boger and Baker[7] have reported that the administration of caronamide will increase the blood penicillin levels by about 8-fold and that this effect can be achieved at any dose level of penicillin.

To determine the clinical usefulness of such a drug two questions must be answered. (1) When administered orally over a long period in amounts that will be tolerated by the patient, does the drug effectively increase the blood penicillin level? (2) Under these circumstances, does the drug produce any renal damage? As far as caronamide is concerned, the question of renal damage seems to have been well answered in the negative. There is no available information of this sort concerning benzoic acid. This report is presented in an effort to answer the above questions.

Methods: The penicillin blood levels were determined by the reductase method of Reid and Brewer.[14] This method gave good reproducible results within the range of blood con-

* Aided by a grant from the Medical School Research Committee, University of Utah, and in part by the Life Insurance Medical Research Fund.

† "Staticin" Caronamide (4 Carboxyphenylmethanesulfonamide) was furnished through the courtesy of Sharpe and Dohme, Inc., Philadelphia, Pennsylvania.

centrations found in our subjects and has the advantage over other methods in that there is a sharp endpoint and one that is easy to read. The method was checked by repeated determination of known concentrations of penicillin in saline and in serum and was also checked against the Rammelkamp method. Under all these circumstances it was found to be adequate. All determinations were carefully controlled for the presence of the so-called "antisubtilis factor".[14] Determinations were usually made within a few hours of the time the blood sample was withdrawn and when this was not feasible the serum was promptly separated and frozen. Blood urea nitrogen levels were determined by the method of Archibald[1] and the endogenous creatinine clearances were determined by the usual Jaffe reaction using a tungstate filtrate.

Plan of Study and Results. *Benzoic Acid*—Nine patients in the Intensive Treatment Center (7 with early syphilis and 2 with central nervous system syphilis) were selected as subjects for this study. All were free from evidence of any other disease, particularly renal or hepatic, except 1 who showed consistently a diminished creatinine clearance without other evidence of disease. At the start it was thought that the patients could be studied while receiving the routine treatment of mapharsen, penicillin and bismuth but it soon was discovered that mapharsen caused a sharp, though temporary, reduction in renal function. Consequently the patients used in this experiment received only penicillin. No attempt was made to limit either fluid or food

TABLE 1.—BLOOD PENICILLIN LEVELS AND BLOOD UREA NITROGEN LEVELS IN PATIENTS RECEIVING 12 GM. OF BENZOIC ACID DAILY

Patient	Average Blood Penicillin Level Units cc.		Average Blood Urea Nitrogen Mgs./100 cc.		Creatinine Clearance cc./min.	
	before Benzoic Acid	after Benzoic Acid	before Benzoic Acid	after Benzoic Acid	Initial	Final
D.R.	0.20	0.17	17	14	57	60
S.V.	0.20	0.10	11	18	82	87
Man.	0.07	0.20	11	18	92	99
Mar.	0.08	0.23	28	27	92	131
A.T.	0.11	0.10	18	9	70	69
An.	0.16	0.09	21	19	144	145
L.H.	0.22	0.28	23	31	115	124
J.V.	0.12	0.14	18	13	94	103
H.P.	0.04	0.09	17.5	14.7	129	82

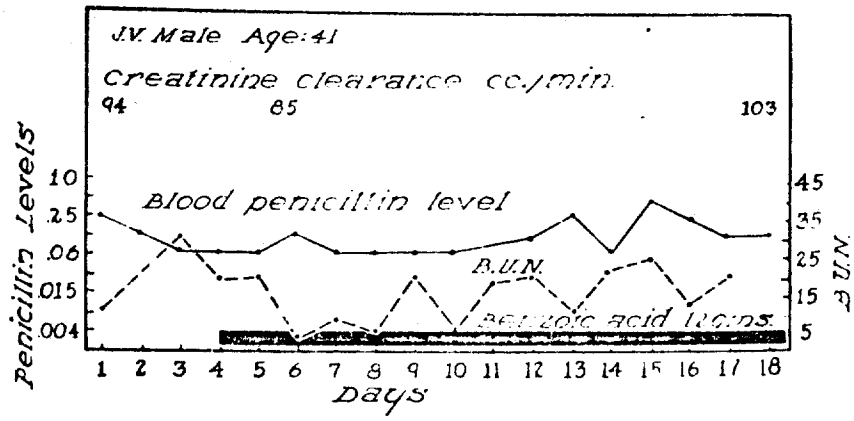

Chart 1.—The blood penicillin level, blood urea nitrogen, and creatinine clearance of 1 patient who received 12 gm. of benzoic acid per day for 14 days. None of these was significantly different from control levels.

intake during the experimental periods. Crystalline penicillin G in saline was administered intramuscularly to all subjects in doses of 30,000 units every 3 hours. The control period was started after the subjects had received penicillin for 18 to 24 hours. Penicillin and blood urea nitrogen levels were determined daily one and one-half hours after the most recent dose of penicillin and the endogenous creatinine clearance was measured at the beginning and end of the experimental period and in some cases on the fifth day as well. After a 4-day control period, 12 gm. of benzoic acid divided into 8 doses was administered orally for the next five days to all the subjects except one, who received this amount of benzoic acid for 14 days.

The results of these observations are summarized in Table 1. It may be seen that of the 9 subjects, there was some increase in the average blood penicillin levels in 3, no essential change in 3, and a lower average level in 3. Individual charts, however, show that the changes were not constant from day to day and that there was really little essential change over the control level. One Patient (J. V.) did show about a 2-fold enhancement but only after receiving benzoic acid for 7 days (Chart 1). The blood urea nitrogen levels showed no consistent alteration beyond the rather wide variation from day to day that is to be expected when the patient is receiving the usual hospital diet. In no case did the endogenous creatinine clearance show any significant change from the control period. Routine urinalysis also failed to show any abnormality.

Caronamide. Twelve patients (5 luetics and 7 convalescent from various infectious diseases) were used as subjects. All were free from evidence of renal or hepatic disease. Blood penicillin levels were determined daily at 1 and 3 hours after an injection of penicillin. All subjects received aqueous penicillin intramuscularly at 3 hour intervals, but did not necessarily receive the same dose. Each individual subject, however, did receive the same amount of penicillin throughout the experiment. After a 2 day control period, the patients were given caronamide in doses varying from 12 to 24 gm. per day in 8 equal doses for the next 3 days. In addition to these short term studies, combined penicillin and caronamide therapy was maintained for at least 1 month in 2 subjects. Each patient received 24 gm. or more of caronamide per day. Penicillin levels

TABLE 2.—BLOOD PENICILLIN LEVELS IN PATIENTS RECEIVING CARONAMIDE DAILY

Patient	Control Period Penicillin cc. blood Av. 1 hr.	Av. 3 hrs.	Dose Caronamide Grams 24 hrs.	While receiving Caronamide for 24 hrs. Penicillin cc. blood at 1 hr.	3 hrs.	48 hrs. 1 hr.	3 hrs.	72 hrs. 1 hr.	3 hrs.
Gi.	0.5	0.12	24	4.0	1.0	16.0	1.0	16.0	2.0
O.	0.5	0	24	0.5	0.12	2.0	1.0	2.0	1.0
Ge.	0.25	0.09	24	0.5	0.06	1.0	0.5	2.0	0.25
Du.	0.18	0	24	1.0	0.25	0.12	0.06	0.5	0.06
J.B.	0.31	0	20	4.0	0.5	1.0	0.12	2.0	1.0
C.P.	0.5	0	20	1.0	0.12	0.25	0	0.25	0.06
Hic.	0.5	0.09	16	0.5	0.12	0.5	0.5	2.0	0.5
D.D.	0.25	0.06	16	0.5	0.06	0.5	0.12	1.0	0.25
Ke.	0.25	0	12	1.0	0.06	1.0	0.12		
St.	0.09	0	12	0.12	0.06	1.0	0.12	0.5	0
Sm.	0.12	0	12	0.06	0	0.12	0	0.5	0
Carl.	0.12	0	12	0.06	0	0.12	0	0.25	0

were determined 2 or 3 times a week. The purpose of this part of the experiment was not so much to determine enhancement of the blood penicillin levels as to evaluate the feasibility of administering caronamide over a long period.

The results of the caronamide study are shown in Table 2. It may be seen that doses of 12 and 16 gm. per day failed, in most cases, to alter the blood penicillin level significantly. Doses of 20 to 24 gm. per day, however, gave this patient it became necessary at times to increase the penicillin dosage. Therefore, the actual increase in blood penicillin levels cannot be ascribed wholly to caronamide. We were particularly interested in one phenomenon, shown in Chart 3. After administration of 24 gm. of caronamide per day for 8 days, the penicillin levels fell off rather sharply and symptoms recurred. When the dose of caronamide was increased to 32 gm. per day the blood penicillin levels became stable and

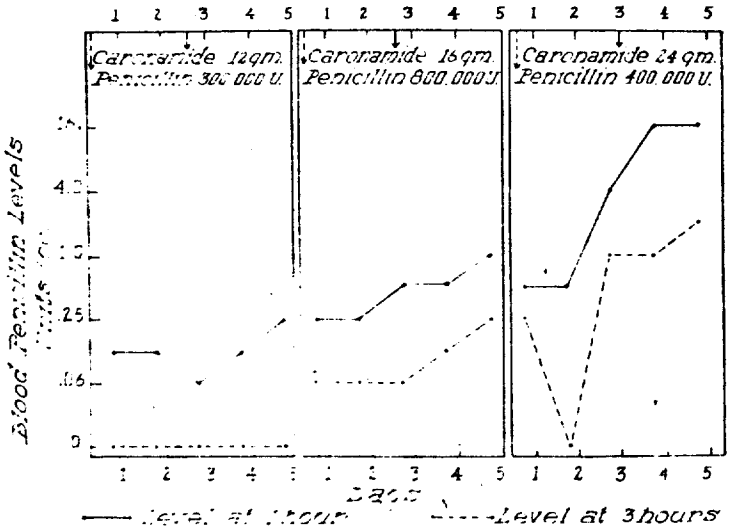

Chart 2.—The effect of various doses of caronamide on the blood penicillin level of individual subjects. The arrows indicate the time at which the administration of penicillin and caronamide respectively was begun.

4 to 16-fold increases in the blood penicillin level (Chart 2). It is of interest to note that in a number of cases the blood penicillin levels were higher 48 and 72 hours after beginning caronamide than at 24 hours. It is noteworthy too that in many cases caronamide produced a greater relative increase in the blood penicillin level at 3 hours than at 1 hour after injection.

The results in 1 of the 2 patients with subacute bacterial endocarditis are shown in Chart 3. In the treatment of remained so during the rest of the time that caronamide was being administered.

This patient, who died on his 96th hospital day, received a total of 1650 gm. of caronamide in 58 days. At autopsy the kidneys were grossly normal and on section showed no abnormality that could be attributed to the drug. This suggests that this drug even in large doses for long periods does not damage the kidneys.

Discussion. From the above data, it is evident that the administration of 12 gm. of benzoic per day has little effect in enhancing the blood penicillin levels. It is true that this quantity is not as great as others have used, but preliminary observations convinced us that this dosage was about all that the usual patient would tolerate over a long period of time. Even with this relatively small amount, about one third of the patients complained of gastric burning and some anorexia. It is entirely possible that had salts and fluids been strictly limited, the levels might have been higher but such restriction over several days is difficult at any time and in the presence of a febrile illness would be undesirable. Boger and Baker[7] used up to 24 gm. of benzoic acid per day and demonstrated no increase of the blood penicillin levels. We were unable to demonstrate any evidence of renal impairment during the administration of benzoic acid over periods up to 14 days and conclude that this drug, in the doses used at least, does not damage the kidney.

Caronamide, on the other hand, when given in doses of 20 gm. a day or more appears to be an effective agent for increasing the blood penicillin levels, and has the additional advantage that no limitation of salt or fluid seems to be necessary. More important, perhaps, is the ability of caronamide to prevent the rapid decline of blood penicillin levels between injections during intermittent parenteral administration.

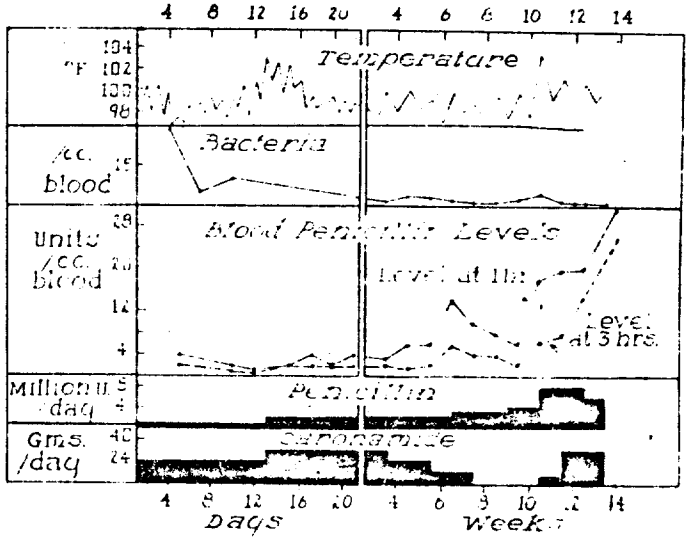

Chart 3.—Summary of data obtained in 1 patient with subacute bacterial endocarditis. In that portion of the chart designated by weeks, the amount of caronamide and penicillin administered is indicated as the average per day for that week.

Note the sharp pre-mortal rise in the blood penicillin level.

We have found this drug to be ordinarily well tolerated by the patients. About one-quarter of the patients complained of mild anorexia which seemed to correlate best with the number of pills they were taking. No severe reactions attributable to caronamide were observed.

We did not determine the blood or

urine caronamide levels of any of the subjects in this series. Such measurements may prove to be useful for adjusting caronamide dosage. We believe, however, that the alteration of the blood penicillin level gives a good index as to the effectiveness of the caronamide dosage and at the same time offers more useful clinical information than the caronamide level. This is well illustrated in Chart 3.

It has been stated by a number of investigators that in the treatment of most of the infectious diseases that respond to penicillin, higher levels are easily obtained by simply increasing the dose of penicillin and that the various enhancing agents are of no great clinical value. In general, we are in accord with this view. However, in certain diseases, particularly subacute bacterial endocarditis and those infections caused by an organism with a relatively high resistance to penicillin, caronamide is a valuable adjuvant to therapy, and combined penicillin and caronamide will result in higher and better sustained levels than can be obtained by the use of any reasonable dose of penicillin alone.

Summary and Conclusions. 1. In the presence of normal fluid and electrolyte intake, benzoic acid was ineffective in enhancing the blood penicillin level. In doses of 12 gm. per day for periods of 14 days or less no measurable renal damage was observed.

2. Caronamide in suitable dosage is an effective agent for increasing and prolonging the blood penicillin concentration.

3. One patient received 1650 gm. of caronamide and showed no damage to the kidneys when these were examined post mortem.

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GROWTH-INHIBITION PRODUCED IN RATS BY THE ORAL ADMINISTRATION OF SODIUM BENZOATE

Effects of Various Dietary Supplements*

ABRAHAM WHITE

Growth inhibition in rats has been produced by the incorporation of a variety of toxic compounds into the diet.[21, 22, 23, 24] Further, it has been possible to demonstrate that stimulation of growth occurs under these experimental conditions when one of the sulfur-containing amino acids, cysteine, cystine, or methionine, is superimposed on the basal diet still containing the growth-inhibitory compound. Under these conditions the animals resume growth at a rate comparable to that observed on the basal diet alone. In addition, other substances, e.g., glutathione and cystine disulfoxide, which are known to stimulate growth in rats stunted by one of the so-called "cystine-deficient" diets, have also been shown to be effective growth stimulators when added to the basal, growth-inhibiting diets. Inasmuch as these growth-promoting dietary supplements exert their action when injected subcutaneously, as well as following oral administration, it has been concluded that their effect is a true metabolic one and not due to a reaction occurring in the intestine prior to absorption.

The specific and unique capacity of the sulfur-containing amino acids, and of substances behaving in nutrition experiments in a manner similar to these amino acids, to stimulate the growth of animals ingesting a growth-inhibitory basal diet has led to the suggestion that the inhibition of the growth of the rat by certain organic compounds "is a manifestation of the production of a specific deficiency in the sulfur-containing amino acids, probably by imposing on the organism an increased requirement for organic sulfur, in the form of cystine or methionine, for detoxication mechanisms."[24] However, although direct proof for the formation of a detoxication product (mercapturic acid) is clearly established for the monohalogen benzenes, and

* From the Department of Physiological Chemistry, Yale University School of Medicine. Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

for naphthalene and anthracene, the diverse chemical structures which have been found to produce similar growth effects and to yield similar experimental results with various types of supplements suggest some caution in interpreting all of the growth data, obtained in this type of experimentation, on the basis of a detoxication mechanism involving the sulfur-containing amino acids. Until actual isolation of the detoxication product has been achieved in the case of each growth-inhibitory chemical compound, the proposed detoxication mechanism in these instances merely remains a working hypothesis. Indeed, as has been already suggested,[24] it may be that the growth-inhibition is not, in all cases, related to a specific detoxication mechanism involving the sulfur-containing amino acids, but that some of these foreign organic compounds may retard cellular metabolism. In this event, growth-stimulating sulfur compounds, i.e., *l*-cystine, *l*-cysteine, *dl*-methionine, *l*-cystine disulfoxide, and glutathione, may then be regarded as general cell metabolic stimulants with a consequent effect on the somatic growth of the organism. The present experiments were designed to test this possibility. It would appear that an experimental approach could be made to this problem by the stunting of young rats with some dietary chemical substance or compound which, by virtue of its nature or structure, could not theoretically be detoxicated by a mechanism involving the sulfur-containing amino acids. Under these experimental conditions, the effects of cystine or methionine supplements on the growth rate should be of considerable interest.

The growth-inhibitory compound chosen for the present study is sodium benzoate. The well-established detoxication of benzoate by conjugation with glycine and with glycuronic acid, and the chemical structure of benzoic acid, make it unlikely that this compound would have a growth-inhibitory effect only because of a relationship to a specific detoxication mechanism involving the sulfur-containing amino acids. Indeed, the results of this investigation have clearly demonstrated that growth-inhibition produced in rats by oral administration of sodium benzoate is alleviated only by glycine and by compounds which appear to be closely related to glycine from a metabolic standpoint, i.e., sarcosine and glycolic acid, and is uninfluenced by dietary supplements of either cystine or methionine. These data are interpreted as further evidence that growth stimulation produced by a dietary supplement, despite the simultaneous presence in the diet of a growth-inhibiting substance, is indicative of the involvement

of a detoxication mechanism and the formation of some product of the conjugation of the growth-stimulating compound, or its metabolic products, with the substance causing the growth retardation. Under these circumstances, growth is resumed because in the presence of adequate quantities of a growth-promoting dietary supplement, the latter fulfills the organism's requirements for this particular substance for both detoxication mechanisms and the synthesis of new tissue. If an amino acid participating in the detoxication reaction is derived solely from the tissue proteins, then obviously the stimulating dietary adjunct functions chiefly as replacement for the losses from the tissue proteins.

During the course of this investigation several other substances have been tested for their growth-stimulating effect in rats stunted by means of a sodium benzoate-containing diet. The compounds studied as supplements were chosen either because they had been demonstrated to exert an influence on benzoic acid detoxication or because they have been suggested as playing a rôle in some aspect of glycine metabolism. After this study was completed, the interest in some of the dietary supplements was greatly diminished, inasmuch as the original claims made for the rôle of certain compounds in benzoic acid detoxication[5] were later withdrawn.[6]

Experimental

The basal diet used was the low-casein ration employed in previous studies from this laboratory.[24] Male rats, at weaning, were placed in individual cages and given this diet *ad libitum*. In addition, each animal received a daily supplement of 400 mg. of dried yeast.* When the animals reached a body weight of approximately 75 to 85 gm., 5 gm. of sodium benzoate (U.S.P. Powder, Merck) were added to each 100 gm. of basal diet. Several lower concentrations of benzoate were tried in early preliminary experiments, but it was found that 5 gm. for each 100 gm. of basal diet was the minimum quantity which could produce marked growth inhibition. After periods varying from 3 to 6 weeks in length on the stunting diet, each supplement was incorporated separately into the benzoate-containing basal ration in order to study its effect on the inhibited growth rate. Following a period of at least 2 weeks, during which time the effect of the supplement was determined, animals which had been

* Product of the Northwestern Yeast Co.

given growth-stimulating supplements were returned to the benzoate-containing basal diet and rats which had received substances ineffective in relieving the growth inhibition were placed upon a benzoate-containing basal diet supplemented with glycine. In this manner each animal served as its own control. The supplements employed were as follows: glycine (Eastman Kodak Co.), *l*-cystine (prepared from hair), sarcosine (Hoffmann-La Roche Co.) *dl*-methionine (Organic Manufacturers, University of Illinois), creatine (Valentine Meat Juice Co.), glycolic acid (Eastman Kodak Co.),

TABLE 1

SUMMARY OF AVERAGE WEIGHT CHANGE AND AVERAGE FOOD CONSUMPTION OF ANIMALS RECEIVING BASAL DIET AND VARIOUS TYPES OF SUPPLEMENTS

*Diet**		*No. of animals in group*	*Average daily weight change gm.*	*Average daily food consumption gm.*
Basal		115	+ 1.3	6.2
Basal + sodium benzoate		106	0.0	3.3
Benzoate-containing basal diet + each of the following growth-promoting dietary supplements:				
glycine	(1.0 gm.)	52	+ 1.0	5.0
sarcosine	(1.0 gm.)	7	+ 0.7	5.6
glycolic acid	(1.0 gm.)	4	+ 0.4	5.1
glycolic acid	(1.5 gm.)	3	+ 0.8	4.9
glycolic acid	(2.0 gm.)	7	+ 0.9	5.3
Benzoate-containing basal diet + each of the following ineffective dietary supplements:				
l-cystine	(1.0 gm.)	6	− 0.1	4.6
dl-methionine	(1.0 gm.)	3	− 0.3	3.7
glycocyamine	(1.0 gm.)	2	− 0.1	4.7
creatine · H_2O	(1.25 gm.)	3	− 1.6	2.1
l-hydroxyproline	(1.25 gm.)	2	− 0.2	4.9
dl-serine	(1.25 gm.)	3	− 0.3	3.7
dl-threonine	(1.0 gm.)	2	− 0.3	4.2
dl-threonine	(2.0 gm.)	3	− 0.2	4.5
sodium sulfate	(1.5 gm.)	2	− 0.8	4.3
sodium citrate	(1.5 gm.)	3	0.0	5.1

* The figures in parentheses indicate the levels at which each dietary supplement was added to each 100 gm. of the benzoate-containing basal diet.

dl-threonine (Organic Manufacturers, University of Illinois), glycocyamine (Hoffmann-La Roche Co.), *dl*-serine (synthetic), and *l*-hydroxyproline (Hoffmann-La Roche Co.). In addition, the effect of sodium sulfate and of sodium citrate supplements was also studied. The purity of the nitrogen-containing supplements was indicated by total nitrogen analyses (micro-Kjeldahl). The purity of the glycolic acid was established by carbon and hydrogen analyses. The inorganic supplements were commercial preparations of analytical reagent quality. The data which have been obtained are summarized in Table 1.

Results

The use of sodium benzoate to inhibit the growth of rats was suggested by Griffith[11] in investigations of the origin of glycine. The latter investigation incorporated sodium benzoate in varying quantities (1.5 to 3.75 per cent) into a basal diet in which the protein was furnished by casein at a 35 per cent level. These diets were fed to young male rats during an experimental period (usually 40 days) in which careful record of growth rate was made. Survival and growth of young rats on diets containing benzoate occurred only when these diets furnished a supply of glycine which was adequate for the detoxication of the benzoate and for the formation of new tissue proteins.

The amount of sodium benzoate used in the present experiments was chosen after a number of preliminary trials. It was desired to produce rather consistent stunting of the large number of rats employed in order that the data might be quite uniform. The addition of 5 gm. of sodium benzoate to each 100 gm. of basal diet produced the desired stunting without any obvious signs of gross toxicity. Indeed, four rats were used as long-time controls and were permitted to ingest the benzoate-containing basal diet for a period of 23 weeks. During this time no evidence of toxicity was observed.* Although the body weights of the animals remained practically stationary dur-

* Although no gross toxicity was apparent in the animals whose data are presented in Table 1, it should be pointed out that a rat was occasionally seen which did not tolerate sodium benzoate ingestion and could not be used. In these animals there was evident a restlessness and irritability, and in a few instances constant tremors involving the whole body were seen. These symptoms are similar to those described by Griffith[11] in rats ingesting sodium benzoate. In no case was diarrhea evident.

ing this period, the rats were still responsive to the superimposition of a growth-promoting supplement, e.g., glycine, on the benzoate-containing basal diet. This responsiveness was manifested as a prompt resumption of growth and a steadily increased growth rate as long as the glycine feeding was continued.

The effectiveness of glycine in stimulating the growth of rats stunted by sodium benzoate is seen from the data in Table 1. The conjugation of glycine with benzoic acid to form hippuric acid has been extensively studied by a number of investigators. The specificity of glycine for this reaction is seen, for example, in the experiments of Griffith and Lewis.[14] The latter investigators observed that glycine when administered (in 3 molecular equivalents) with benzoic acid to rabbits almost doubled the rate of excretion of hippuric acid as measured by the urinary output in the first 6 hours after feeding. None of the other amino acids studied affected the rate of excretion in this way. Further, similar data have been reported in recent publications by Abbott and Lewis.[1, 2] The present growth data are also in complete accord with those of Griffith.[11]

It will be seen from Table 1 that sarcosine, incorporated into the benzoate-containing basal diet at the same level as glycine, produced a growth-stimulating effect comparable to that observed with the latter amino acid. The effects of sarcosine supplements on benzoate-containing basal diets were first examined by Gordon and Jackson,* whose unpublished preliminary data strongly suggested the capacity of this N-methylated amino acid to function in lieu of glycine in stimulating the growth of animals stunted by means of benzoate feeding. Further confirmation of these findings was sought in the present study in view of the ability of the N-monomethyl derivatives of a number of the essential amino acids to replace the unsubstituted amino acids for purposes of growth in the diet of the young white rat.[8, 9, 10, 16, 17, 20] While the present investigation was in progress, two laboratories independently confirmed the biological conversion of sarcosine to glycine. Abbott and Lewis[1] reported that sarcosine, when fed with benzoic acid, increased the rate of excretion of hippuric acid by the rabbit. Inasmuch as the increased rate of excretion of conjugated benzoic acid was not due to the formation of N-methyl-

* Private communication. Grateful acknowledgment is made to Dr. Gordon for discussion of some aspects of the unpublished work conducted in collaboration with Dr. Jackson. The experiments of the latter investigators are referred to in: Gordon, W. G., and Jackson, R. W.: J. Biol. Chem., 1939, *110*, 151, cf. p. 163.

hippuric acid, the evidence was interpreted as indicative of the conversion of sarcosine to glycine. Further proof of this conversion was obtained in the experiments of Bloch and Schoenheimer,[3] who administered sarcosine labeled with heavy nitrogen (N^{15}) to adult white rats and found that glycine containing isotopic nitrogen was deposited in the tissue proteins. Moreover, Borsook and Dubnoff[7] have reported that sarcosine could furnish glycine for the synthesis of glycocyamine by rat kidney slices. The recent publication of Handler, Bernheim, and Klein[15] is of interest in connection with the conversion of sarcosine to glycine. These investigators have demonstrated the presence in liver tissue of several species (rat, rabbit, and guinea-pig) of an enzyme capable of oxidatively demethylating sarcosine to glycine.

The influence of dietary glycolic acid on the growth of rats stunted by benzoate feeding was examined carefully because of the demonstration by Griffith[12] that the addition of sodium glycolate to a toxic benzoate diet afforded rats practically the same protection as that furnished by glycine itself. Milhorat and Toscani[19] have reported a metabolic effect of administered glycolic acid resembling that of glycine on the creatinuria of patients with progressive muscular dystrophy. In the present study, the addition of 1 gm. of glycolic acid to each 100 gm. of the benzoate-containing basal diet produced a definite, though not striking, stimulation of growth. As the level of glycolic acid in the diet was increased (1.5 gm. per 100 gm. of benzoate-containing diet), the growth stimulation was marked and when the amount of the glycolic acid supplement was twice the level (2 gm.) at which glycine (1 gm.) was tested, the growth-stimulatory effect approximated closely that seen with glycine. It may be added that a further increase in the quantity of the glycolic acid supplement (2.5 gm. per 100 gm. of benzoate-containing diet) did not further enhance the growth rate but, indeed, resulted in a manifestation of toxic symptoms with growth of the animals ceasing entirely. While these experiments were in progress, Professor Howard B. Lewis of the University of Michigan stated in a private communication that experiments in his laboratory had demonstrated the effectiveness of glycolic acid in augmenting the rate of hippuric acid excretion in rabbits given sodium benzoate. The full details of these studies are now available.[2] It is of considerable interest that on the basis of equivalent weights glycolic acid was found to be less effective than was glycine or sarcosine in increasing the rate of

hippuric acid excretion after benzoate feeding. The data were interpreted as evidence of the ability of the rabbit to convert glycolic acid to glycine to a limited extent. The present growth data are in good agreement with the findings of Abbott and Lewis.

In contrast to the above growth-promoting supplements, each of the other compounds tested proved completely incapable of stimulating the growth of animals stunted by benzoate feeding. The sulfur-containing amino acids, *l*-cystine and *dl*-methionine, so effective in alleviating other types of growth inhibition, had no influence on the growth retardation produced by the benzoate-containing basal diet. The demands of the organism for glycine, or compounds which function like this amino acid in metabolism, would seem to be a manifestation of a specific need for glycine for the detoxication of benzoic acid. This observation lends further weight to the suggestion that the capacity of the sulfur-containing amino acids (and of substances behaving in nutrition experiments in a manner similar to the sulfur-containing amino acids) to stimulate the growth of animals ingesting a diet containing a foreign, growth-inhibiting substance is evidence of the functioning of these amino acids in processes concerned with the detoxication of the growth-inhibiting compounds and not a result of a generalized stimulation of cellular metabolism.

The demonstrated formation of glycocyamine from glycine[3, 7] led to the testing of the former compound in the present study in order to determine whether the reverse transformation, i.e., glycocyamine to glycine, might occur. The inability of glycocyamine to promote growth under the experimental conditions employed would appear to argue against the conversion of glycocyamine to glycine in the organism. Creatine supplements were also ineffective in promoting growth of animals ingesting the benzoate-containing basal diet. The ineffectiveness of creatine is in conformity with the negative results with glycocyamine and is also in support of the recent study of Bloch, Schoenheimer, and Rittenberg[4] which demonstrates the biological resistance of the creatine molecule. The latter, although being continually synthesized, is not significantly degraded.

Hydroxyproline and serine were studied in the present investigation because of the preliminary report[5] that the synthesis of a substance which behaves like hippuric acid occurred when either of these two amino acids was added to the liver and kidney tissues of several species. The inability of these amino acids to stimulate growth in the benzoate-stunted animals is more in agreement with facts dis-

closed in a later publication,[6] to the effect that although either hydroxyproline or serine added to rat liver slices does give rise to a substance which behaves like hippuric acid in the analytical procedure employed, definite proof remains to be obtained that the compound being formed in the presence of either hydroxyproline or serine is actually hippuric acid. The growth data indicate that no conversion of either of these amino acids to glycine occurs under the present experimental conditions.

The possibility of a conversion of threonine to glycine was examined because of the suggestion that β-hydroxy amino acids are oxidized at the beta carbon atom; under these circumstances glycine might arise from threonine in metabolism. However, threonine added at two different levels to the basal benzoate-containing diet had no growth-promoting effect. Sodium sulfate and sodium citrate supplements were tested because of the reports that both sodium sulfate[13] and sodium citrate[18] protect animals from the toxic effects of dietary benzoate. No evidence of decreased toxicity, *as measured by change in growth rate*, was seen when either of these sodium salts was added to the benzoate-containing basal diet.

Summary

1. Inhibition of growth of the young white rat has been produced by the addition of sodium benzoate to a low-protein basal diet. The ability of various supplements to affect the growth rate of the animals was studied by the addition of each supplement to the benzoate-containing basal diet.

2. Under these experimental conditions, glycine or sarcosine supplements produced a prompt stimulation of the growth rate. Glycolic acid, superimposed on the benzoate-containing basal diet in *optimal* quantities, resulted in a prompt increase in growth rate; the growth-promoting effect of glycolic acid under these experimental conditions is somewhat less marked than is that of either glycine or sarcosine.

3. A number of other supplements were studied either because they had been suggested as playing a rôle in benzoic acid detoxication or because they might throw light on the question of whether growth-inhibition by a foreign toxic dietary constituent was a manifestation of an inadequate supply in the organism of a substance required for the detoxication of the growth-inhibiting substance or whether the

diminished growth rate was evidence of a generalized inhibited cellular metabolism. All of the supplements in this group were without effect on the growth rate of benzoate-stunted animals.

4. It is concluded that the data obtained are further evidence for the suggestion that the inhibition of the growth of the rat by certain dietary foreign compounds is a manifestation of the production of a deficiency in the normal metabolic substance or substances specifically concerned with combination with the growth-inhibitory substance to form a detoxication product.

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Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 35 MAY 1957 NUMBER 5

THE EFFECT OF VARIOUS DENATURANTS ON THE HAEMOGLOBINS OF ADULT AND CORD BLOOD

I. COMPARATIVE RATES OF DENATURATION[1]

F. D. WHITE AND AUDREY KERR

Abstract

At room temperature and pH 8.5, the effects of concentrated solutions of urea, nicotinamide, guanidine, sodium benzoate, and sodium salicylate upon the haemoglobins of adult and cord blood have been followed spectrophotometrically. In all cases the reaction curves were similar to those of alkali denaturation, but differed in the rates of reaction and failure to show any marked difference in rate between adult and cord blood. In no instance was the rate for cord blood greater than the corresponding rate for adult blood. The velocity constants for these experimental conditions have been calculated.

Except in the case of the nicotinamide reaction, absorption curves of the reaction products before and after reduction with sodium dithionite showed differences from the corresponding curves of alkali denaturation at pH 11.9. It is suggested this may indicate varying degrees of denaturation.

Introduction

When a dilute solution of sodium hydroxide is added to a haemolyzate of red cells, the bright red color is changed to brown, very rapidly if the cells are from human adult blood, but more gradually if fetal or cord blood has been used. It has been shown by Haurowitz (5) that this color change is due to denaturation and since it is accompanied by a change in the absorption spectrum, the course of the denaturation can be followed spectrophotometrically. Resistance to alkali denaturation is characteristic of fetal haemoglobin and has been used to determine the amount of this constituent in cord blood and in the blood of the newborn (1, 10, 14, 17). Haurowitz (6) has also shown that while the absorption spectra of haemoglobins from different animal species are not appreciably different from that of the human, wide variations exist in the rates of their denaturation by alkali.

Little attention has apparently been paid to the effect of denaturants other than sodium hydroxide upon adult and fetal haemoglobin, although Gardikas *et al.* (2) investigated the action of urea and sodium salicylate upon adult and fetal methaemoglobin and reported the latter to be denatured by urea at a faster rate than the former. This apparent reversal of the generally accepted "refractory" nature of the fetal pigment suggested the present study to us.

[1]Manuscript received November 30, 1956.

Contribution from the Department of Biochemistry, University of Manitoba, Winnipeg, Canada. This work was aided by a grant from the National Research Council of Canada.

The denaturing effects of urea, guanidine, nicotinamide, sodium salicylate, and sodium benzoate upon the haemoglobins of adult and cord blood have been investigated at room temperature (approximately 23° C.) and the rates of denaturation determined. All experiments were carried out at a pH of 8.5 in which the denatured protein remained in solution. Urethane was also investigated, but precipitation invariably occurred before denaturation was complete.

Experimental

General Procedure

In all cases fresh samples of oxalated blood (adult or cord) were centrifuged, the supernatant plasma removed, and the cells washed three to four times with 0.9% sodium chloride solution. Approximately 0.3 ml. of the washed cells were then added to 20 ml. of 0.01 *M* Sorensen's glycine–NaCl–NaOH buffer of pH 8.5, well shaken, and filtered. A 0.5 ml. quantity of this solution was transferred to a 10 mm. absorption cuvette containing 2.5 ml. of the same buffer solution, mixed, and the optical density read in either a Beckman DU or a Hilger Uvispek Spectrophotometer, at 578 mμ. The reading was then adjusted to an absorbancy of 0.7–0.9 by suitable dilution of the original solution, and this formed the control. The denaturing reagents were dissolved in buffer solution to give solutions with a final pH of 8.5, 2.5 ml. were mixed with 0.5 ml. of the haemoglobin solution in the cuvette, and spectrophotometric readings were taken immediately and at suitable time intervals which varied with the denaturant used. The reaction was considered to be complete when the final reading showed no appreciable change in 24 hours. At this point the contents of the cuvette should no longer show the absorption bands at 540 and 578 mμ, typical of oxyhaemoglobin.

Denaturants

In each case preliminary experiments were carried out to determine the concentration of denaturant which at the desired pH would have the greatest effect and still maintain the protein in solution. With urea, the most suitable concentration was found to be 50% and this, when mixed in the absorption cuvette with the haemoglobin solution, gave a final concentration of approximately 7 *M*. With nicotinamide, sodium benzoate, and sodium salicylate, solutions of 50%, 30%, and 20% respectively were used; the final concentrations being approximately 3.4 *M*, 1.7 *M*, and 1 *M*. To get a guanidine solution of pH 8.5, it was necessary to dissolve the hydrochloride in a much stronger and more alkaline buffer than in the previous cases. By utilizing a 0.1 *M* glycine–NaCl–NaOH buffer of pH 12.4, a 40% solution of guanidine-HCl was obtained at the required pH. This gave a final guanidine concentration of approximately 3.5 *M*. In this last case it was obviously impossible to use the haemoglobin buffer solution as control owing to the difference in pH. Consequently the control value was taken as the reading of the haemoglobin-guanidine solution immediately after mixing, the rate of denaturation being sufficiently slow to render the error a negligible one.

Calculation

The calculation was carried out according to the method previously reported for sodium hydroxide denaturation (17), the percentage of undenatured haemoglobin (H) at time t being represented by the equation:

$$H = 100\,(D_t - D_0)/(D_a - D_0)$$

where D_a is the density reading of the control and therefore represented 100% undenatured haemoglobin; D_t is the reading at time t; D_0 the reading after complete denaturation. The curves were constructed by plotting the logarithmic values for H against time.

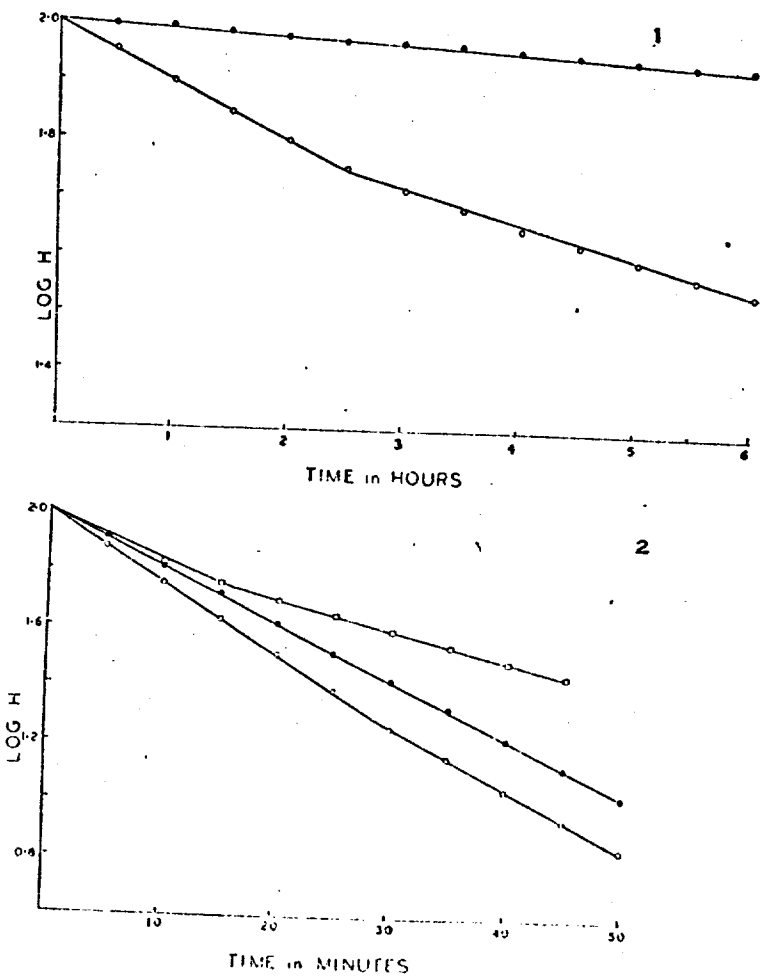

FIG. 1. Effect of urea upon adult and cord blood (H = percentage of undenatured haemoglobin). O - - - - O, adult; ● - - - - - - ●, cord.

FIG. 2. Effect of nicotinamide and guanidine upon adult and cord blood (H = percentage of undenatured haemoglobin). Nicotinamide: O - - - O, adult; ● - - - - - ●, cord. Guanidine: □ - - - - - □, adult and cord.

TABLE I

RATES OF THE REACTIONS OF VARIOUS DENATURANTS WITH THE HAEMOGLOBINS OF ADULT AND CORD BLOOD

K = velocity constant: where reaction curve indicates two rates, K_1 represents initial velocity, and K, residual velocity

Denaturant	Adult Hb		Cord Hb	
	K (per sec.)	K_1 (per sec.)	K (per sec.)	K_1 (per sec.)
Urea	4.1×10^{-5}	6.6×10^{-5}	1.1×10^{-5}	—
Guanidine	3.8×10^{-4}	6.1×10^{-4}	3.8×10^{-4}	6.1×10^{-4}
Nicotinamide	7.9×10^{-4}	9.6×10^{-4}	7.4×10^{-4}	—
Sodium benzoate	2.5×10^{-3}	3.9×10^{-3}	1.9×10^{-3}	3.1×10^{-3}
Sodium salicylate	3.7×10^{-3}	6.4×10^{-3}	3.7×10^{-3}	5.3×10^{-3}
Sodium hydroxide (pH 11.9)	—	*5.7×10^{-2}	5.9×10^{-4}	—

*Since 95% of haemoglobin is denatured in 1 minute, the initial rate is the significant value.

Results

The results are shown in Figs. 1–3. In all cases the points represent mean values obtained from six to eight experiments carried out under the same conditions, the straight lines being drawn according to the method of least squares. Fig. 3 also shows typical curves of sodium hydroxide denaturation at pH 11.9, prepared for purposes of comparison (17). The comparative velocity constants calculated according to the formula: $K = [1/(t_2 - t_1)] \ln(H_{t_1}/H_{t_2})$ are shown in Table I. Where definite breaks in the curves indicated changes in the rate of reaction, the constant for the initial velocity (K_1) was calculated separately. The reaction rates showed a large variation, urea being the slowest acting, and sodium salicylate the fastest, although the latter rate was still markedly less than that for alkali denaturation of adult blood. With the exception of urea, very little difference was found in the reaction rates for adult and cord blood, and even with urea the difference was small in comparison with that for alkali denaturation (see Fig. 3). In no case, however, was there found any evidence of cord haemoglobin reacting at a faster rate than the adult form, and since the cord blood used generally contained about 20% of the adult component, the reaction rates for true fetal haemoglobin must be definitely less than the figures given.

Discussion

It has been shown that when strong concentrations of denaturants are added to haemoglobin solutions, a reaction occurs characterized by a progressive fall in the optical density measured at 578 mμ, similar to the decline observed when haemoglobin undergoes denaturation with sodium hydroxide. The respective rates of reaction, however, differ markedly with the reagent, and moreover do not show the pronounced difference between adult and cord haemoglobins characteristic of alkali denaturation. The question then arises: can these reactions be considered "denaturations" in the same sense of the term ?

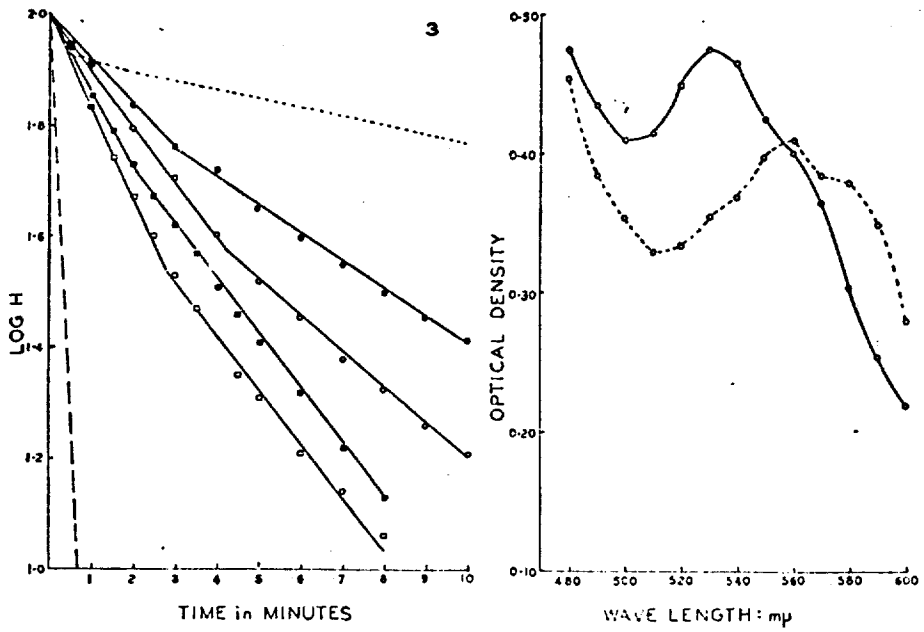

FIG. 3. Effects of sodium benzoate and sodium salicylate upon adult and cord blood, with curves of alkali denaturation at pH 11.9 for comparison. (H = percentage of undenatured haemoglobin). Sodium benzoate: O- - - - - -O, adult; O————O, cord. Sodium salicylate: □ ————□, adult; □- - - - - - □, cord. Alkali denaturation:—·—·—·—, adult; . . . . . , cord.

FIG. 4. Typical absorption curves of urea denaturation product before (O————O) and after (O. . . . . O) reduction with sodium dithionite.

When oxyhaemoglobin is denatured by sodium hydroxide the absorption bands at 578 and 540 mμ disappear and the product gives an absorption curve similar to, but not identical with, that of alkaline haematin (8). When this product, which we have referred to as alkaline globin haematin (17), is reduced by sodium dithionite ($Na_2S_2O_4$) it is converted to a haemochrome (the conjugation of ferroporphyrin with base (9)), with characteristic bands at 530 and 560 mμ. This denatured globin haemochrome can react to give stable haemochromes with basic substances, and in fact the pyridine and nicotinamide derivatives have been used as a means of determining the total haem pigments in blood (11, 12, 16). In the experiments now reported the products of the nicotinamide and guanidine reactions gave absorption curves similar to that of alkaline globin haematin, but whereas after addition of dithionite the nicotinamide product gave a typical haemochrome absorption curve, the guanidine curve showed only a plateau effect at 550–570 mμ. The absorption curve from the reaction with urea differed from the other two in showing a peak at 530 mμ somewhat similar to the spectrum which Hanowitz (4) ascribed to denatured globin haematin. This curve together with that of the corresponding reduced product is shown in Fig. 4. On addition of ammonia the guanidine and urea reduced products were both converted into typical haemochromes. The reactions with sodium benzoate and sodium salicylate

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INFLUENCE OF FOOD PRESERVATIVES AND ARTIFICIAL COLORS ON DIGESTION AND HEALTH.

IV.—BENZOIC ACID AND BENZOATES.

INTRODUCTION.

In the continuation of the work described in Parts I to III of this bulletin, experiments were conducted, according to the general plan already described, to determine the effects of benzoic acid and benzoates upon health and digestion. This investigation is of special importance because of the opinion held by many manufacturers, food officials, and consumers that benzoic acid and benzoates are probably the least harmful of the preservative substances employed. It is believed that for this reason there has been a very large increase in the use of these preservatives in the last few years with a corresponding decrease in the amount of other preservative substances employed. It has also been claimed that there can be no reasonable objection to the use of benzoic acid by reason of its natural occurrence in many food products, either in traces or in considerable quantities. Among the products cited the cranberry occupies the most prominent position because of the notable amount of benzoic acid it contains. These considerations, however, had no determining influence on the choice of this substance for the experimental work, inasmuch as it was included in the original scheme which was prepared before the work reported in Part I was begun.

The same principles which guided the organization of the work as described in Part I were followed in the present instance. Upon the selection of the members of the hygienic table each man was subjected to a thorough medical examination of the character already described. [illegible] was admitted to the table who was suffering from any organic [illegible] who manifested any tendency to hereditary disease, or who [illegible] within the year previous to the beginning of the [illegible]

The delay which has attended the presentation of this report for publication has been due to several causes. First, the great burden of collating the data, condensing the analytical tables, and checking the data for accuracy, required, as is usual in such cases, a large amount of time and expert labor. There were also a number of points brought out in the investigations which required further study of the question, both experimentally and in consulting authorities thereon.

Another reason for the delay consisted in the fact that various representations were making on the part of manufacturers and others respecting the effect upon the industries using benzoic acid should the conclusions reached in this report receive executive and judicial confirmation. It was thought advisable, therefore, to give ample time to the industries involved to experiment with methods of manufacture looking to the elimination of objectionable preservatives. Investigations were also undertaken by this Bureau in collaboration with the manufacturing interests along the same line. Results of these investigations have shown that there is not a single article of food which has been commonly preserved by means of benzoic acid or benzoate of soda which cannot be preserved and offered to the consumer in perfect condition without the aid of any chemical preservative. This fact has been completely demonstrated in the case of cider and grape juice, mince-meat, jelly, jams, catsups, preserves, and other articles of the same character, and there seems, therefore, to be no longer any industrial reason for delaying publication even if the former necessity for such delay be admitted.

It is believed that the distribution of the results of this investigation at the present time will neither work hardship to any manufacturing interest nor interfere in any way with any legitimate business. At the same time it will indicate to the manufacturer, as well as to the consumer, the important truth that the use of benzoic acid or benzoate of soda as a preserving medium is not without danger. that its effects are always injurious or tend to injury, and that its exclusion from food products is desirable not only in order to conform to the food and drugs act, but also for hygienic reasons.

The greater care which is required in the manufacture of food products without the use of benzoic acid or benzoate of soda, necessitating the use of a higher quality of raw material, will place the industries which would otherwise use these preservatives in foods on a better plane, and secure for their products a greater consumption.

SERIES VIII.

ADMINISTRATION OF THE PRESERVATIVE.

In Table I are recorded the dates of the periods and subperiods during which this experiment was conducted. A preliminary or relaxation period of one month elapsed between the close of Series VII and the beginning of Series VIII, the subjects being the same in both series with the exception of No. 1.

TABLE I.—*Dates of periods and subperiods in Series VIII.*

Period and subperiod.	Date of beginning.	Date of ending.
[illegible]	[illegible]	[illegible]

In Table II is given a schedule of the administration of the preservative. The sodium benzoate used is calculated in the table as benzoic acid. The preservative was given in all cases in capsules, as experience had shown this to be the best method from every point of view of administering a substance of this nature.

In the first preservative subperiod there was given to Nos. 1 to 6, inclusive, one gram of benzoic acid per day; to Nos. 7 to 12, inclusive, one gram per day with the exception of the first day when only 0.9 gram was given. During the second preservative subperiod 1.5 grams of benzoic acid were given each day to each member. In the third preservative subperiod 2 grams, and in the fourth 2.5 grams were given each day, with the exceptions noted in the table. There were several cases during the fourth subperiod when the preservative, by reason of its ill effects and for other causes, had to be withdrawn. The maximum quantities of benzoic acid, therefore, given during the entire preservative period are 35 and 34.9 grams, and only three men were able to take these amounts. In all of the other cases it was necessary to withdraw a portion or all of the preservative for the reasons already stated.

TABLE II.—*Schedule of administration of preservative, Series VIII.*

[In capsules.]

Period and date.	Benzoic acid.						Sodium benzoate (expressed as benzoic acid).					
	70	70	64	59	51	59	69	60	62	56	67	68
	No. 1.	No. 2.	No. 3.	No. 4.	No. 5.	No. 6.	No. 7.	No. 8.	No. 9.	No. 10.	No. 11.	No. 12.
	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.	Gms.
First subperiod:												
April 21, 1904	1	1	1	1	1	1	0.9	0.9	0.9	0.9	0.9	0.9
22, 1904	1	1	1	1	1	1	1	1.0	1.0	1.0	1.0	1.0
23, 1904	1	1	1	1	1	1	1	1.0	1.0	1.0	1.0	1.0
24, 1904	1	1	1	1	1	1	1	1.0	1.0	1.0	1.0	1.0
25, 1904	1	1	1	1	1	1	1	1.0	1.0	1.0	1.0	1.0
Total per individual	5	5	5	5	5	5	4.9	4.9	4.9	4.9	4.9	4.9
Second subperiod:												
April 26, 1904	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
27, 1904	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
28, 1904	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
29, 1904	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
30, 1904	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Total per individual	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
Third subperiod:												
1, 1904	2	2	1	2	2	2	2	2	2	2	2	2
2, 1904	2	2	0	2	2	2	2	2	2	2	2	2
3, 1904	2	2	0	1.5	[a] 1	2	2	2	2	2	2	2
4, 1904	2	2	0	2.5	1	2	2	2	[a] 2	2	2	2
5, 1904	2	2	0	2	2	2	2	2	2	0	2	2
Total per individual	10	10	1	10.0	8	10	10	10	10	8	10	10
Fourth subperiod:												
May 6, 1904	2.5	2.5	0	2.5	2	0	1.5	2.5	0	0	2.5	0
7, 1904	2.5	0	0	2.5	1	0	2.5	2.5	0	0	0	0
8, 1904	2.5	0	0	2.5	0	0	2.5	2.5	0	0	0	0
9, 1904	2.5	0	0	2.5	0	0	0	2.5	0	0	0	0
10, 1904	2.5	0	0	2.5	0	0	0	2.5	0	0	0	0
Total per individual	12.5	2.5	0	12.5	3	0	6.5	12.5	0	0	2.5	0
Total per individual for entire preservative period	35.0	25.0	13.5	35.0	22.5	22.5	28.9	34.9	22.4	20.4	24.9	[illegible]

[a] Took preservative, but became sick immediately afterwards.

EXCRETION OF HIPPURIC AND BENZOIC ACID.

In Table III are given the results of the determinations of hippuric and benzoic acid in the urine during the entire time of observation.

METHOD FOR DETERMINING HIPPURIC AND BENZOIC ACID IN THE URINE.

The method employed was that described by Bunge and Schmiedeberg.[a] The essential features of the method are as follows:

Make alkaline 100 to 200 cc of urine with sodium carbonate, evaporate to dryness and extract the residue with alcohol. Completely distill off the alcohol, make the remaining water solution acid with hydrochloric acid and extract at least five times with acetic ether. Wash the acetic ether extract with water and then evaporate.

[a] Arch. exp. Path. Pharmakol., 1876, 6: 225; also Analyse des Harns, Neubauer and [illegible]

Purify the hippuric acid and benzoic acid, crystallize, and weigh. Separate the hippuric acid from benzoic acid by means of petroleum ether; the difference in weight of the crystalline forms is benzoic acid.

The method is not as exact as could be desired and presents many difficulties, particularly in the case of concentrated urines, where a considerable quantity of fatty and resinous matter is removed by the acetic ether employed to extract the benzoic and hippuric acids. As in other determinations the analyses were made on composited samples.

Hippuric acid occurs as a normal constituent of human urine in amounts varying from 0.1 gram to 1 gram per day (Analyse des Harns, Neubauer and Vogel), and in some cases after eating freely of vegetables and fruit, especially plums, cranberries, etc., it may be more than 2 grams per day.[a] In the ordinary mixed diet the average quantity of hippuric acid eliminated is given as 0.7 gram per day.

Hippuric acid is the chief nitrogenous constituent in the urine of herbivora. This is explained by the fact that animals feeding wholly on vegetable foods consume a large amount of aromatic substances, which, by oxidation, as toluol (cinnamic acid), or by reduction, as quinnic acid, are converted into benzoic acid, or substances containing the benzene nucleus, and then, by combination with glycocoll, are converted into hippuric acid and excreted as such.

The formation of hippuric acid in the human organism is therefore associated with the formation of benzoic acid. It has been proven conclusively, both synthetically and by feeding experiments, that hippuric acid is formed as the resulting product of the union of benzoic acid, or a substance containing the benzene nucleus, and glycocoll. Thus any substance or material taken with the food which contains the benzene nucleus or is capable, by oxidation or reduction, of being converted into benzoic acid will unite with glycocoll, which is derived from the protein metabolism within the body, to form hippuric acid.

There are also a few cases where the benzoic acid is derived solely from protein. Salkowski, Meissner, Shepard, and others found hippuric acid in the urine of starving dogs, also in dogs' urine after a diet consisting entirely of meat. The benzoic acid in these cases evidently originated from the putrefaction of protein in the intestines.

The amount of hippuric acid eliminated is influenced, first, by the amount of glycocoll present, and, second, by the amount of benzoic acid formed. If there is sufficient glycocoll formed during the digestion of proteids to combine with the benzoic acid, then all will

GENERAL CONCLUSIONS.

From a careful study of the data in the individual cases and of the summaries of the results, it is evident that the administration of benzoic acid, either as such or in the form of benzoate of soda, is highly objectionable and produces a very serious disturbance of the metabolic functions, attended with injury to digestion and health.

As in the case of boric acid, salicylic acid, and sulphurous acid, this injury manifests itself in a number of different ways, both in the production of unfavorable symptoms and in the disturbance of metabolism. These injurious effects are evident in the medical and clinical data which show grave disturbances of digestion, attended by phenomena which are clearly indicative of irritation, nausea, headache, and in a few cases vomiting. These symptoms were not only well marked, but they were produced upon healthy individuals receiving good and nourishing food and living under proper sanitary conditions. It is only fair to conclude, therefore, that under similar conditions of administration of benzoic acid or benzoate of soda in the case of weaker systems, or less resistant conditions of health, much more serious and lasting injury would be produced.

It was also noticed that the administration of benzoic acid and benzoate of soda was attended with a distinct loss of weight, indicative of either a disturbance of assimilation or an increased activity in those processes of the body which result in destruction of tissue. The production of a loss of weight in cases of this kind must be regarded as indicative of injurious effects.

The influence of the benzoic acid and benzoate of soda upon metabolism was never of a character indicative of a favorable change therein. While often the metabolic changes were not strongly marked, such changes as were established were of an injurious nature. It is evident that the administration of these bodies, therefore, in the food tends to derange metabolism in an injurious way.

An important fact in connection with the administration of these bodies is found in the efforts which nature makes to eliminate them from the system. In so far as possible the benzoic acid is converted into hippuric acid. There is a tendency usually manifested, however, to retain the benzoic acid in the body for a notable length of time, and this is much more marked in the case of benzoate of soda than in the case of benzoic acid.

While the administration of both these bodies, therefore, is undoubtedly harmful, the injurious effects are produced more rapidly in the case of benzoic acid than they are in the case of benzoate of soda; the data, however, will show that the total harmful effect produced in the end is practically the same in both cases, hence,

there appears to be no reason for supposing that the administration of the preservative in the form of benzoate of soda can be justified by any argument relating to the less injurious effect thereof upon health.

The occurrence of microscopic bodies in the urine is undoubtedly increased under the administration of benzoic acid in both forms, thus showing conclusively the tendency to stimulate the destructive activities of the body.

Coming to the final consideration of all of these different phases of the subject, there is only one conclusion to be drawn from the data which have been presented and that is that in the interests of health both benzoic acid and benzoate of soda should be excluded from food products. This conclusion is reached independently of any consideration of the conditions which it is alleged surround the processes of manufacture and which result in the demands of manufacturers to be allowed to continue the use of these substances. This is a subject which must be discussed from an entirely different point of view and has no bearing whatever upon the general conclusions which have been reached, namely, that both benzoic acid and benzoate of soda are bodies which, when added to foods, are injurious to health.

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